To my husband,
Kirtikumar Seth,
for all his support

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Immunochemical Studies On The Calcitonin Gene Peptides

Rashmi Seth, B.Sc., M.Phil. (University of London)

Thesis submitted to the UNIVERSITY OF LONDON
for the degree of
DOCTOR of PHILOSOPHY

Endocrine Unit,
Department of Chemical Pathology,
Royal Postgraduate Medical School.
London W12 ONN

1989

Abstract

The programme of work described in this thesis is concerned with the development and application of novel immunoassays for the measurement of peptides from the calcitonin gene and is divided into three major sections.

Section I (chapter 1) reviews the literature on calcitonin (CT) - a peptide hormone concerned with long term skeletal maintenance during periods of calcium stress; calcitonin gene-related peptide (CGRP) - a novel peptide with a potent vasodilatory action; and katacalcin (KC) - a peptide with unknown biological actions, from their discovery to the present day.

Section II (chapter 2) describes the development and applications of novel immunoassays for CT and CGRP using existing polyclonal antibodies.The method of enzyme amplification has been incorporated into the methodology to enhance the sensitivity of the resulting assays. The results obtained are compared and contrasted with the established radioimmunoassays (RIA's).

Section III (chapters 3, 4 and 5) presents the utilisation of the monoclonal antibody technology to perform immunochemical studies on the human CT (hCT) and human CGRP (hCGRP). Chapter 3 details the production and characterisation of monoclonal antibodies raised against hCT and hCGRP. Epitope mapping for anti-calcitonin antibodies confirmed earlier findings that the carboxyl-terminal of the molecule was more immunogenic than the rest of the molecule and that the presence of an amidated proline residue was required for immunological activity.

In chapter 4, the development and applications of a monoclonal antibody-based enzyme-immunometric assay (EIA) for hCT are presented. The assay, which is one of the most sensitive assays for hCT available to date, was applied to the measurement of the hormone in plasma of normal volunteers, post-menopausal women and patients with medullary carcinoma of the thyroid (MTC). Earlier findings that women have lower CT levels than men was confirmed using the EIA and it was shown that after the menopause, CT levels drop dramatically. In the same chapter, the isolation and partial characterisation

of circulating immunoreactive forms of hCT, hCGRP and KC in MTC plasma is detailed using size-exclusion chromatographic techniques. The immunoreactive monomeric CT-like material eluting at approximately 3000 MW identified by both the monoclonal based-EIA and the polyclonal based-RIA was tested for biological activity. It was found to be biologically active in the isolated rat osteoclast bioassay system. The high molecular weight material eluting in the void volume region of the column (approx MW > 60,000) was found to be biologically active after urea dissociation, indicating that CT bound to albumin-like material is potentially biologically active.

Finally, the significance of this work in relation to previous and future studies is discussed in the last chapter (chapter 5).

Acknowledgements

I am greatly indebted to my supervisor, Professor Iain MacIntyre, whose support and guidance made these studies possible.

I wish to thank Dr Samia Girgis and Dr Anne Kehely for critical reading of the manuscript. I wish to acknowledge Professor Colin Self, Dr Sunil Wimalawansa, Dr Harish Datta and Dr Mone Zaidi for their collaboration and helpful discussions. I am especially thankful to Dr Zaidi for performing the isolated osteoclast bioassays.

I would also like to acknowledge Professor Dominique Bellet, Professor Claude Bohuon and Dr Philippe Motte of the Institut Gustave-Roussy, Villejuif, France, for extending to me the facilities in their department and for their collaboration. I am especially thankful to Dr Motte for his personal help during my stay in France.

I also wish to thank Dr David Bates and Dr James Fuller of Novo Biolabs, Milton Road, Cambridge, for the preparation of enzyme-labelled analytes and antibodies and Mrs Patricia Hill for radiolabelling hCT for use in RIA.

I would like to thank Mrs Brenda Salvage for her skilful assistance in the preparation of manuscripts for publication.

The financial support of the Medical Research Council is gratefully acknowledged.

I am indebted to my family for their support throughout this study.

List of Abbreviations

AEIA	amplified enzyme-immunoassay
BSA	bovine serum albumin
CT	calcitonin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CPM	counts per minutes
DNA	deoxyribonucleic acid
cDNA	complementary DNA
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HAT	hypoxanthine aminopterin thymidine
i-hCT	immunoreactive human CT
IgG	immunoglobulin G
IRMA	immunoradiometric assay
MW	molecular weight
MTC	medullary carcinoma of the thyroid gland
O/N	overnight
O.D.	optical density
RIA	radioimmunoassay
RNA	ribonucleic acid
mRNA	messenger RNA
RRA	radioreceptor assay
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel-electrophoresis
UV	ultra violet
PPF	plasma purified fraction
RA	radio assays

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SECTION I

Chapter 1: Literature Review

Summary

The calcitonin gene encodes a family of peptides: calcitonin, calcitonin gene-related peptide (CGRP) and katacalcin. Calcitonin (CT) is a hormone concerned with skeletal maintenance during periods of calcium stress. CGRP is a neuropeptide and a potent vasodilator. The biological actions of katacalcin, if any, are still unknown. The existence of CGRP and katacalcin were predicted in the rat by recombinant DNA technique. Human CGRP and katacalcin were subsequently isolated and sequenced proving the presence of the predicted peptides in man.

More recently, a second CT/CGRP gene has been reported in both rat and man, commonly referred to as the beta-gene. This gene has been shown to encode another CGRP molecule. There is also a CT-like exon in this gene. However it is unlikely to be expressed in man. There is a weak structural homology between CT and CGRP which explains the ability of the peptides to interact at each other's receptors. CT receptors are found on the osteoclasts whereas those for CGRP are present at certain sites in the nervous system and throughout the vascular system.

Amylin, a novel 37 amino acid peptide is the latest member of the CGRP family. It has 46% homology with beta - CGRP and 43% with alpha - CGRP.

This chapter reviews the literature on some relevant aspects of CT and CGRP in terms of their physiological activities.

CHAPTER 1 : Literature Review

1.1 Calcitonin

1.1.1. Discovery

Plasma calcium is regulated with great precision. In the past, parathyroid hormone was considered, in the presence of vitamin D, the only important regulator of plasma calcium. In 1958, McLean proposed a direct negative feedback mechanism, in which parathyroid hormone secretion would be stimulated by hypocalcaemia and inhibited by hypercalcaemia. Parathyroid hormone acted too slowly to be able to account for small changes in calcium homeostasis and therefore it was suggested by the same author that the short-term regulation might involve the pool of readily exchangeable calcium ions in the bone. It was suggested by Rasmussen in 1961 that a simple system which only involved bone and parathyroid hormone may lead to wide variations in plasma calcium levels.

During the studies carried out by Copp, Cameron, Cheney, Davidson and Henze (1962) on the direct hormonal control of parathyroid functions, it was observed that hypercalcaemic perfusion of the thyroid-parathyroid complex in dogs produced a fall in plasma calcium which occurred earlier and was greater than that following total thyroparathyroidectomy. They postulated the existence of a new, fast acting calcium lowering hormone which they named 'calcitonin'.

In 1963, Kumar, Foster and MacIntyre confirmed the existence of calcitonin (CT) in dogs, but noted that the perfusion of isolated parathyroids was not technically possible in these animals and the origin of calcitonin had yet to be proved. Hirsch, Gauthier and Munson (1963) reported that ablation of parathyroids in the rat by electrocautery caused a greater fall in plasma calcium than did their surgical removal. Cautery of the thyroid alone produced a large hypocalcaemic effect similar to that caused by the injection of acid extracts of the thyroid tissue. This suggested that the hypocalcaemic factor was of thyroidal origin, and it was named 'thyrocalcitonin' to distinguish it from Copp's

calcitonin.

Later, perfusion experiments in goats carried out by Foster, Baghdiantz, Kumar, *et al.*(1964) clearly established thyroid as the source of calcitonin.The name calcitonin (CT) was generally used in preference to thyrocalcitonin since it was found to be present in the ultimobranchial body of submammalian vertebrates (Tauber, 1967; Moseley, Matthews, Breed, *et al.*,1968) and also in the thymic and parathyroid tissue in man (Galante, Gudmundsson, Matthews, *et al.*,1968).

1.1.2. Isolation of calcitonin

CT was first isolated from the pig thyroid (Baghdiantz, Foster, Edwards, *et al.*,1964). Early attempts to isolate human CT (hCT) from the normal thyroid failed. It was realised that medullary thyroid carcinoma contained 5000 times more CT than the normal thyroid (Cunliffe, Black, Hall, *et al.*,1968), and using this tissue as the starting material, the peptide was isolated in monomeric (CT-M) and dimeric (CT-D) forms (Riniker, Neher, Maier, *et al.*, 1968) and sequenced (Neher, Riniker, Maier, *et al.*,1968). Since then a large number of mammalian and sub-mammalian CT's have been isolated, sequenced and synthesised (Girgis, Galan Galan, Arnett, *et al.*, 1980).

1.1.3. Structure-Activity relationship

CT is composed of a single chain of amino acid residues with a disulphide bridge between positions 1 and 7, and an amidated proline residue in the C-terminal. There is considerable variation in amino acid sequence, between the calcitonins of different species (for review see Zaidi, Breimer and MacIntyre, 1987). There are 8 'invariant' residues at positions 1, 4, 5, 6, 7, 9, 28 and 32. Six of the conserved residues are located in the N-terminal ring structure and residues 10-27 include no invariant amino acids at all (see table 1).

The termini have been found to be juxtaposed in spatial configuration (Byfield, Clark, Turner, Foster, and MacIntyre, 1972) and appear to be important for biological activity since cleavage of the 1-7 disulphide bond in

porcine and hCT results in the loss of biological activity (Sieber, Brugger, Kamber, *et al.*, 1970 and Rittel, Maier, Brugger, *et al.*, 1976) as does the deletion of C-terminal proline amide. Replacement of the disulphide bond (S-S) by an ethylene linkage (C-C) in eel calcitonin (asu^{1-7}) does not affect the biological activity (Morikawa, Muniketa, Sakakibara, Noda and Otani, 1976). Oxidation of methionine at position 8 in hCT destroys biological activity (Sieber *et al.*,1970) while oxidation of porcine and ovine methionine at position 25 causes no change. Furthermore, methionine is totally absent in the highly active salmon I and salmon III. Therefore, it is possible that the variable middle portion of the molecule may control potency and duration of action. Table 2 shows the effect of modification on the biological activity of CT.

The higher biological potency of salmon and ultimobranchial CT's compared to mammalian CT's may be explained by the fact that the salmon CT has a greater affinity for receptors in kidney and bone than porcine CT (Marx, Woodward and Aurbach, 1972), but other factors include greater resistance to metabolic degradation and consequently longer half-life in the circulation (Habener, Singer, Deftos, Neher and Potts, 1971; De Luise, Martin and Melik, 1970).

It has been suggested that the conformational flexibility of the molecule can significantly affect its biological activity (Epand, Epand, Orlowski, Seyler and Colesscott, 1986). Replacement substitutions of salmon CT with amino acids having less bulky side-chains (valine with glycine at position 8, or leucine with alanine at position 16) have been found to produce more potent analogues. This has been shown to be due to an increasingly flexible molecule with a reduced tendency to form helical structures.

Amino-acid Position	hCT	alpha-hCGRP	beta-hCGRP	ratCGRP	katacalcin
1	Cys	Ala	Ala	Ser	Asp
2	Gly	Cys	Cys	Cys	Met
3	Asn	Asp	Asn	Asn	Ser
4	Leu	Thr			Ser
5	Ser	Ala			Asp
6	Thr	Thr			Leu
7	Cys	Cys			Cys
8	Met	Val			Arg
9	Leu	Thr			Asp
10	Gly	His			His
11	Thr	Arg			Arg
12	Tyr	Leu			Pro
13	Thr	Ala			His
14	Gln	Gly			Val
15	Asp	Leu			Ser
16	Phe	Ser			Met
17	Asn	Arg			pro
18	Lys	Ser			Gln
19	Phe	Gly			Asn
20	His	Gly			Ala
21	Thr	Gly			Asn
22	Phe	Val			
23	Pro	Val	Met	Val	
24	Gln	Lys			
25	Thr	Asn			
26	Ala	Asn	Ser	Asp	
27	Ile	Phe			
28	Gly	Val			
29	Val	Pro			
30	Gly	Thr			
31	Ala	Asn			
32	Pro	Val			
33	NH_2	Gly			
34		Ser			
35		lys		Glu	
36		Ala			
37		phe			
		NH_2	NH_2	NH_2	

Table 1: Amino acid sequence of peptides from the calcitonin gene

Structural modifications	Species	Effect on Biological Activity
Deletion		
C-terminal	All	loss
N-terminal		loss
Clevage		
1-7 disulphide bridge	porcine human	loss
Substitution		
disulphide bridge with ethylene group (C-C)	eel	no effect
valine with glycine at position 8	salmon	increased potency
leucine with alanine	salmon	increased potency
Oxidation		
methionin at position 8	human	loss
methionin at position 25	salmon	no effect

Table 2: The effect of modification of the amino acid sequence on the calcium lowering effect in young rats

1.1.4. Calcitonin in other species

CT is fundamentally a neuropeptide and the hypothesis put forward by Pearse and Polak in 1971 that the C-cells originate from the neural crest was soon confirmed for both man and submammalian vertebrates (Le Douarin and Le Lievre, 1972).

It is now known that whilst migrating from the neural crest during embryonic development, C-cells often concentrate in regions other than the thyroid and ultimobranchial body. The part or gland in which there is a high concentration of C-cells also varies in different species (MacIntyre and Stevenson, 1980; Galan Galan, Rogers, Girgis, *et al.*, 1981a; Galan Galan, Rogers, Girgis and MacIntyre, 1981b).

In the neural complexes and alimentary tract of *Ciona, Ascidella, Branchiostoma,Styela* and *Maxine*, a CT-like molecule has been identified (Girgis, 1980). This molecule resembled human and not salmon CT. This shows that hCT is not only ancient but highly conserved. It has been suggested that the gene producing hCT in primitive protochordates may have duplicated during evolution (Perez-Cano, Girgis and MacIntyre, 1982). In other species, there is also considerable evidence for gene duplication (Archer, 1980).

1.1.5. Physiological actions of calcitonin

The main target tissues for CT are bone and kidney, although it has been suggested that CT may also play a role in the nervous system.

1.1.5.1. Action on bone

Early experiments using isotopes led to the observation that CT acted by inhibition of bone resorption (Milhaud, Perault and Moukhtar, 1965). *In vitro* studies using bone cultures confirmed the above observations and also showed that calcitonin inhibited parathyroid induced bone resorption (Friedman and Raisz, 1965; Aliapoulios, Goldhaber and Munson, 1966). Urinary excretion of

hydroxyproline, an index of bone resorption, was markedly reduced in rats following administration of CT (Martin, Robinson and MacIntyre, 1966 and Aer, 1968).

Perfusion studies in the isolated cat tibia (MacIntyre, Parsons and Robinson, 1967) showed the direct action of CT on bone.

Further *in vitro* studies (Kallio, Garant and Munkin, 1972) suggested that the inhibition of bone resorption caused by CT was due to a rapid loss of ruffled borders of osteoclasts. This observation was confirmed by Holtrop, Raisz and Simmons, 1974 who also observed that CT decreased the ruffled border area in osteoclasts stimulated by parathyroid hormone with subsequent reduction in the release of labelled calcium from pre-treated foetal bones. High affinity binding sites for CT have been identified in rat calvarial membranes (Marx, Woodward and Aurbach, 1972) and have been shown to possess a several fold higher affinity for salmon CT than for mammalian CT's. Enhanced cyclic AMP production by the peptide has been demonstrated in rat calvarial membranes (Klein and Raisz, 1971) and in isolated bone cells (Wong and Cohn, 1975). These effects were additive to, but did not potentiate, those produced by parathyroid hormone suggesting that CT and parathyroid hormone act on separate receptors via a second messenger.

Studies by Chambers and Dunn (1983) showed the direct inhibitory action of CT on the isolated osteoclast. They first demonstrated that CT directly inhibits osteoclastic motility and spreading (Chambers and Magnus, 1982; Chambers and Moore, 1983). They also showed that the peptide inhibits resorption of cortical bone by isolated osteoclasts (Chambers, Revell, Fuller and Athanasou, 1984; Chambers, McSheehy, Thompson and Fuller, 1985 and Zaidi, Chambers, Bevis *et al.*,1986). Prostaglandins and dibutyaryl cyclic AMP produce the same effects as CT (Chambers and Ali, 1983). These effects have formed the basis of sensitive bioassays developed for direct quantification of osteoclastic activity (Zaidi, Chambers, Bevis *et al.*, 1986).

1.1.5.2. Action on kidney

It was shown by Gudmundsson, MacIntyre and Soliman (1966) that the plasma calcium lowering effect of CT in rats was not impaired significantly by nephrectomy, indicating that it did not promote the renal excretion of calcium. However, infusion of CT into parathyroidectomised rats led to a dose dependent phosphaturia (Robinson, Martin and MacIntyre, 1966), but the action appeared to be a minor one and not responsible for the observed fall in plasma phosphate (Robinson, Martin, Matthew and MacIntyre, 1967). No phosphoturic effect of CT was observed in either the pig (Russell and Fleisch, 1968) or the dog (Clark and Kenny, 1969).

The action of CT on kidneys has been shown to be mediated by cyclic AMP. Membrane preparation from rat renal cortex and outer medulla responded to stimulation by salmon CT with increased production of cyclic AMP (Marx, Woodward and Aurbach, 1972) and CT also appeared to act on a cell population distinct from that sensitive to parathyroid hormone or vasopressin.

1.1.5.3. Action on gastrointestinal tract

The actions of CT on the gastrointestinal tract are not of major physiological importance. The hypocalcaemic effect of CT was still evident after removal of the gastrointestinal tract in rats (Aliapoulios, Savery and Munson, 1965) and calcium absorption from isolated gut loops was not affected by CT (Robinson, Matthews and MacIntyre, 1969).

Infusion of CT into normal volunteers stimulated the intestinal secretion of sodium, potassium, chloride and water but had no effect on calcium (Gray, Beiberdorf and Fordtran, 1973) and this action might be linked to the diarrhoea often observed in patients with medullary carcinoma of the thyroid where there are high circulating CT levels. Becker, Reeder, Scurry, and Thompson (1974) reported that CT decreased gastrin and gastric acid secretion in normal subjects after meals.

1.1.5.4. Effect of calcitonin on the nervous system.

In several species, including man, CT has been shown to produce profound effects on physiological, behavioural and metabolic actions mediated by the central nervous system. The basic mechanisms for these effects appear to be the modulation of neuronal excitability (Twery and Moss, 1985). Centrally administered CT decreases food consumption, gastric acid secretion and affects patterns of intestinal motility (Bueno, Fioramonti and Ferre, 1983).

Intravenous or intraventricular CT administration inhibits basal and stimulated levels of prolactin, (Oligiati, Guidobono, Lusisette, Netti, Bianchi and Pecile, 1981). During lactation, the CT levels are elevated and the inhibitory effect of CT on prolactin is most pronounced (Oligiati, Netti, Guidobono and Pecile, 1982). These effects appear to be mediated via dopaminergic neurones in the hypothalamus (Clementi, Nicoletti, Patacchioli, Prato, Patti, Matera and Scapagnini, 1983). Intraventricular CT administration increases the pain threshold in rabbits (Pecile, Perrie, Braga and Oligiati, 1975). Analgesic effects have been found to be mediated via serotonergic nuclei in the mid-brain raphe (Clementi, Amico, Roxas, *et al.*, 1985) and not via opiate receptors (Braga, Ferri, Santagostino, Oligiati and Pecile, 1978).

1.1.6. Plasma calcitonin

1.1.6.1. Normal levels

The physiological role of CT is incompletely understood, but since it acts by inhibiting bone resorption, it would be expected that its major effects on plasma calcium would be at those times at which bone resorption is contributing significantly to the maintenance of plasma calcium, that is, when rapid growth is taking place. Thus in normal subjects, injection of hCT has no effect on plasma calcium (Foster, Joplin, MacIntyre, Melvin and Slack, 1966; Martin and Melick, 1969), whereas in children and adults with increased bone resorption from thyrotoxicosis or Paget's disease of bone it lowers plasma calcium (McCredie, Dixon, Martin, Melick, Rotenberg and Shipman, 1971; Bijvoet, Van der

Sluys Veer & Jansen, 1968).

CT levels are elevated during pregnancy and lactation (Stevenson, Hillyard, MacIntyre, Cooper and Whitehead, 1979). This observation led to the suggestion that the physiological role of CT may be that of the protection of the maternal skeleton. In normal adults , the plasma i-hCT* levels have been reported to be higher in males than in females (Hillyard, Stevenson and MacIntyre, 1978) and decline with age in both sexes (Samaan, Anderson and Adam-Mayne, 1975; Deftos, Weisman, Williams, *et al.*, 1980).

1.1.6.2. Levels in medullary thyroid carcinoma (MTC)

MTC is a tumor of the CT -producing C-cells of the thyroid gland. It was first recognised by Hazaard, Hawk & Crile in 1959 as a distinct pathological entity that could be distinguished from other thyroid tumors by its histological features which include uniformity of cell types, a moderate number of mitoses as compared to the undifferentiated or anaplastic carcinoma, a uniform arrangement of cell sheets separated by stroma and the presence of amyloid in the tumor. The suggestion that the C-cells might be the cells of origin of MTC was proved to be correct in 1968 when several investigators demonstrated by bioassay techniques the presence of abnormal CT concentrations in plasma and tissue of patients with MTC (Cunliffe, Black, Hall, *et al.*, 1968; Meyer & Abel-Bari 1968; Milhaud, Tubiana, Parmentier and Coutris, 1968; Melvin and Tashijian 1968). In fact it was the concentration of this hormone in MTC that permitted the isolation, purification and sequencing of hCT (Neher, Riniker, Zuber, Rittel and Khant, 1968). These findings were confirmed by specific RIA of hCT in tumor and plasma by Clark, Boyd, Byfield and Foster, 1969, and histochemical and immunohistochemical studies (Kalina, Foster, Clark and Pearse, 1969).

MTC comprises about 7-10% of thyroid carcinomas. A range of 3.5 - 11% was recorded in a review by Ljungberg (1972). The occurrence of medullary carcinoma as the most frequent type with a familial association was established by Schimke & Hartmann (1965). It is inherited as an autosomal dominant and Williams (1965) reported the association of MTC with phaeochromocytomas.

* i-hCT= immunoreactive human calcitonin

The term "multiple endocrine neoplasia type II" was designated by Steiner, Goodman & Powers (1968) who reported the association of medullary thyroid carcinoma, parathyroid adenopathy which is now identified as type I (Wermer 1963; Ballard, Frame and Hartsock, 1964).

The diagnostic value of plasma immunoreactive hCT measurement in conjunction with radiological and scintigraphic techniques is well established in the diagnosis and treatment of MTC (Cunliffe, Black, Hall, *et al.*, 1968; Clark *et al.*, 1969). Most patients with this tumor have elevated CT in basal plasma samples (Melvin, Tashijian and Miller, 1972; Samaan, Hill, Bedero and Schultz 1973). However, during the early stages of tumor growth, the basal levels of CT may be within the normal range and stimulation tests of CT secretion are necessary to establish the early diagnosis, particularly among relatives of an index case when the familial form is suspected.

1.1.6.3. Stimulation tests

1.1.6.3.1. Calcium

Intravenous infusion of calcium has been the most widely used method to stimulate secretion of CT in MTC. In studies carried out by Deftos, Powell, Parthemore and Potts in 1973, calcium infusion at rate of 3-5 mg/Kg of body weight /h for 2-4 hours has consistently produced elevated hCT levels during or immediately after the infusion period. This method was unsuitable since calcium infusion often led to the elevation of blood calcium levels for several hours, which can produce nausea and vomiting. The length of the infusion necessitated admission of the patient to a hospital for this purpose which is not a feasible procedure where large numbers of patients are to be screened.

A shorter infusion of 150 mg calcium for 5-10 minutes was adopted and did cause significant elevation of hCT. However, the degree of stimulation was not as marked as with the standard calcium infusion.

1.1.6.3.2. Pentagastrin

Peptides containing the carboxyl-terminal tetrapeptide sequence of gastrin have also been shown to be potent stimuli for hCT secretion. The administration of the synthetic gastrin analogue, pentagastrin, when injected intravenously at doses of 0.5mg/kg produces a dramatic increase in plasma hCT some 2 minutes after injection (Hennessy, Wells, Ontjes and Cooper, 1974). It is widely used as a provocative agent for hCT secretion in patients with MTC.

Common side effects are epigastric distress and malaise after the injection which usually persists for 1-2 minutes.

1.1.6.3.3. Alcohol

It was observed by Cohen, MacIntyre, Graame-Smith and Walker, 1973 that the social ingestion of alcohol consistently precipitated flushing and diarrhoea in a patient with MTC. Oral administration of 50ml of whisky was effective in stimulating hCT release when screening families of patients with MTC (Dymling, Ljungberg, Hillyard, Greenberg, Evans and MacIntyre, 1976). The hCT levels rise within 15 minutes and the test has the advantage that whisky is given orally.

1.1.6.3.4. Other Stimulants

Glucagon can stimulate CT secretion but it is not a reliable stimulus since its effect can be variable (Deftos, 1974). Glucagon can also stimulate catecholamine release which could be potentially dangerous in patients with associated phaeochromocytoma. As in the case of glucagon, Deftos *et al.*, (1973) noted that gastrin is not a reliable secretagogue for CT in MTC.

1.1.7. Ectopic secretion of calcitonin

Several cases of ectopic calcitonin production in patients with oat cell lung carcinoma have been reported (Silva, Becker, Primac, Doppman and Snider, 1973). Oat cell bronchogenic carcinoma taken from a patient wth hypercalcaemia

and maintained in proliferative cell culture has been shown to secrete immunoreactive CT indistinguishable from monomeric CT (Coombes, Hillyard, Greenberg and MacIntyre, 1974). Similarly, one monolayer of breast carcinoma cells released immunoreactive CT for 10 weeks. The production of CT by oat cell bronchogenic carcinomas may be explained by the APUD theory (Pearse, 1968) as the cells are derived from neural crest origin. The most likely explanation for the production of immunoreactive CT by breast carcinoma tissue which is not embryologically derived from neural crest tissue, is the gene derepression theory (Gelhorn, 1963), whereby the genetic information within cells which is unrelated to the phenotype, that is, the genes previously repressed are once again active.

Elevated plasma CT levels in patients with non-thyroid tumors have been reported by several groups. Coombes *et al.*, (1974) reported a study of 46 patients with non thyroid carcinomas. Levels were elevated in 21 of whom 8 had oat cell carcinomas and 8 breast cancer. In these patients, the immunoreactive plasma CT was indistinguishable from monomeric CT. In another study by Milhaud, Calmette, Taboulet, Jullienne and Moukhtar (1974) all patients with oat cell carcinoma and 50% of those with carcinoid tumors had elevated hCT.

1.1.8. Calcitonin levels in osteoporosis

Osteoporosis is characterised by a reduction in bone mass in the skeleton, associated with an increased risk of fracture. The bone mass at any age is determined by three factors (Riggs and Melton, 1986). They are the bone mass at maturity, the age at which bone loss begins and the rate at which it proceeds. The peak bone mass at maturity is regulated by a number of hormonal and non-hormonal factors. The factors which contribute to age-related bone loss include menopause, decreased physical activity and decline in calcium absorption with age. In addition to these factors, other conditions associated with the development of secondary osteoporosis are amenorrhoea, anorexia nervosa and elite athletics. The association of these three conditions with osteoporosis probably results from oestrogen deficiency.

Oestrogen has an important role in maintaining the skeletal integrity in the female. The mechanism of action is not yet clear. Oestradiol receptors have been detected in the bone but the apparent absence of oestradiol receptors in osteoclasts and the lack of effects of physiological concentration of oestrogen on bone resorption *in vitro* suggest that the effect of oestrogen on bone may be indirect (Francis, Peacock, Aaron, *et al.*, 1986) and at least two major indirect mechanisms of action have been proposed.

The first suggests that postmenopausal women have a deficient reserve of CT which allows increased bone resorption and that oestrogen replacement restores CT to normal levels , thus protecting the skeleton (Stevenson, Abeyasekera, Hillyard, *et al.*,1981). However, studies on CT secretion after oestrogen replacement have produced contradictory results (Chesnut, Baylink, Sison, Nelp and Roos, 1981; Tiegs, Body, Wahner, *et al.*, 1985) and there is doubt about the mode of action of oestrogen on bone.

In the second hypothesis, oestrogen is said to protect the skeleton through an increase in calcium absorption from the gut, brought about by increased plasma calcitriol levels (Gallagher, Riggs and De Luca, 1980).

However, despite this controversy, there is good evidence that oestrogen treatment reduces bone resorption, decreases or prevents the loss of cortical and trabecular bone, and reduces the incidence of fracture in postmenopausal osteoporosis (Riggs and Melton, 1986). Recently CT has been found to be as effective as oestrogen in the treatment of postmenopausal osteoporosis (MacIntyre, Stevenson, Whitehead, *et al.*,1988).

Other hormonal and non-hormonal factors involved in the pathogenesis of osteoporotic bone loss have been reviewed by Selby and Francis (1988).

1.1.9. Biosynthesis of calcitonin

The first direct evidence that peptide hormones are synthesised initially as part of a large precursor molecule or 'prohormone' emerged from 'pulse-chase' studies *in vitro* (Steiner, Cunningham, Spigelman and Aten, 1967; Kemper, Habener, Potts and Rich, 1972). Roos, Okano and Deftos (1974) studied the CT biosynthesis in trout ultimobranchial cells by following the incorporation of ^{14}C-leucine into cell proteins using immunochemical and chromatographic methods. They detected two main forms of calcitonin, of approximate molecular weights (MW) 7000 and 3000. These forms appeared to exhibit a precursor-product relationship.

Due to the sparse distribution of the CT secreting C-cells in the mammalian thyroid the studies on biosynthesis were hindered. The idea that the precursor is present was supported by the finding of heterogeneous high MW calcitonin present in the plasma of patients with a C-cell tumor known as medullary thyroid carcinoma (MTC) (Singer and Habener, 1974; Deftos, Roos, Bronzert and Parthemore, 1975; Sizemore, Heath and Larson, 1975) and MTC tissue extracts in MTC-exposed cell culture medium (Lichtenberger and Singer, 1975; Goltzman and Tischler, 1978). In order to investigate the existence of a precursor for CT, mRNA studies were carried out.

1.1.10. Cell-Free Translation Studies of calcitonin mRNA

Many *in vitro* systems have been described which can translate heterogeneous messenger RNA. These include cell-free reticulocyte lysate (Lockard and Lingrel, 1969), Krebs III ascites tumor (Matthews, Osborn and Lingrel, 1971) and wheatgerm system (Roberts and Paterson, 1973). Whole egg cells of the toad *xenopus laevis* have also been used for this purpose (Gurdon, Lane, Woodland and Marbaix, 1971).

The wheatgerm system has the advantage of a low background of endogenous protein synthesis in the absence of added mRNA and has been used to investigate biosynthesis of a number of mammalian peptide hormones. The mRNA extracted and purified from the endocrine tissue has been shown to direct

synthesis of precursor to parathyroid hormone (Kemper, Habener, Mulligan, Potts and Rich, 1974) and insulin (Chan, Kevin and Steiner, 1976) in this system. The translation incubations were performed in the presence of radiolabelled aminoacids and their products identified by immunoprecipitation and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The main immunoprecipitable translation product characterised in the above studies appeared to represent the pre-prohormone in which the prohormone is preceded by a hydrophobic N-terminal extension of about 25 aminoacid residues. Unlike prohormone, the pre- prohormone precursor form is only evident in cell-free systems, which lack the post-translational processing capabilities of intact cells. In the signal hypothesis of Blobel and Dobberstein (1975) this hydrophobic leader sequence was postulated to facilitate the translocation of nascent secretory proteins across the endoplasmic reticular membrane. Translation of mRNA encoding the fish pre-proinsulin in the presence of canine microsomal vesicles resulted in the appearance of the authentic proinsulin which was found to be segregated into the luminal space of the vesicles (Shields and Blobel, 1977). These observations suggested that a specific protease was associated with the microsomal membranes which cleaved the leader sequence after it had guided the nascent polypeptide chain across the membrane. Furthermore, the secretory processing mechanism appeared to be highly conserved in view of the heterologous nature of the components used in this system. Similar mechanisms may also be operating in the case of biosynthesis of CT.

The *In Vitro* translation of mRNA coding for precursor to CT has also been reported. These studies mainly utilised mRNA derived from MTC tissue, as well as from the normal thyroid. Van der Donk,Van Dam, Goudswaard, Hackeng and Lips (1976) and Lips, Van der Sluys Veer, Van der Donk, Van Dam and Hackeng (1978) described the translation of unfractionated RNA in whole cells, followed by analysis on non-denaturing PAGE, in each case, yielding a primary product of 65,000 MW. Translation products directed by RNA extracted from several hormone secreting tumors could all be precipitated by anti-CT antisera as

well as by the respective antiserum to the primary hormone secreted by each tumor. These authors suggested that a common, high molecular weight precursor for peptide hormones might exist with processing to different products in each tissue. No independent confirmation of these findings has yet been reported, although Lumsden, Ham and Ellison (1980) have described the characterisation of an ectopically produced CT of MW 40,000. All other studies were carried out by cell-free translation of polyadenylated mRNA with analysis of the product by the denaturing SDS-PAGE method. Estimates for the size of the primary immunoprecipitable product were in good agreement ranging from MW 14,500 to 21,000. The actual MW values obtained appeared to depend on the experimental conditions rather than the source of tissue for mRNA or the choice of cell-free system.

In studies by Goodman, Jacobs and Habener (1979) and Jacobs, Potts, Bell and Habener (1979) addition of microsomal membranes to translation incubations resulted in the partial disappearance of the MW 15,000 translation product, with concomitant appearance of a MW 11,000 to 12,000 product, suggesting cleavage of pre-procalcitonin to procalcitonin. However, Allison, Hall, MacIntyre and Craig (1981) noted that in addition to the primary translation product of 21,000, other human CT precursor forms of MW 19,000, 18,000 and 15,000 were also synthesised particularly when the Mg^{++} concentrations were suboptimal for the production of 21,000 MW. This may have been due to the residual post translational processing capability present in wheatgerm and reticulocyte lysate cell-free system (Pascall, Boulton, Parker, Hall and Craig, 1981). Mg^{++} concentrations that were suboptimal for translation may have been optimal for the activity of proteolytic processing enzymes and may account for the MW 14,000 to 17,000 values obtained for calcitonin precursor in the earlier cell-free translation studies.

Allison and colleagues also noted the high abundance (10%) of calcitonin coding mRNA's in the total polyadenylated mRNA isolated from human MTC. These levels were consistent with the very high level of calcitonin produced by these tumors.

Jacobs, Lund, Potts, Bell and Habener (1981) reported that the translation of rat MTC mRNA yielded a larger precursor (MW 17,000) in the presence of microsomal vesicles than in their absence (MW 15,000). The larger product showed binding to concanavalin A-sepharose and was cleaved to a MW 12,000 by an enzyme of the endoglycoside group. They concluded that the MW 17,000 precursor was glycosylated procalcitonin and that the only potential site of glycosylation was the asparagine residue sequence type Asn-X-Thr or Asn-X-Ser, (Struck, Lennarz and Brew, 1978) at position 3 of the CT sequence.

The primary structure of a large portion of a cloned cDNA complementary to mRNA encoding the rat calcitonin precusor had already been determined by this group (Jacobs, Lund, Potts, Bell and Habener,1981b).

Jacobs and colleagues speculated that glycosylation might play a role in determining the specificity of pre-secretory processing. In support of their findings, Baylin, Wieman, O'Neil and Roos (1981) observed that from 5 - 20% of the immunoreactive calcitonin extracted from human MTC and small cell carcinoma of the lung appeared to be glycosylated. Glycosylated CT with MW up to 27,000 has been detected in extracts of serially transplanted rat MTC (O'Neil, Birnbaum, Jacobson and Roos, 1981). A scheme for the biosynthesis of hCT is shown in figure 1.

1.1.11. Application of recombinant DNA technique to CT

1.1.11.1. Introduction

The techniques of recombinant DNA cloning in bacteria plasmid (Cohn, Chang, Boyer and Helling, 1973) and nucleotide sequence analysis (Maxam and Gilbert, 1977) developed so far have provided an approach of greater understanding of the eukaryotic genes and their products (Douglass, Civelli and Herbert, 1984). Nucleotide sequences coding for the precursors of peptide hormones of previously known amino acid sequence have been reported. This approach is as follows: the isolation of polyadenylated mRNA from hormone producing tissue is carried out and made certain that it can direct the synthesis of

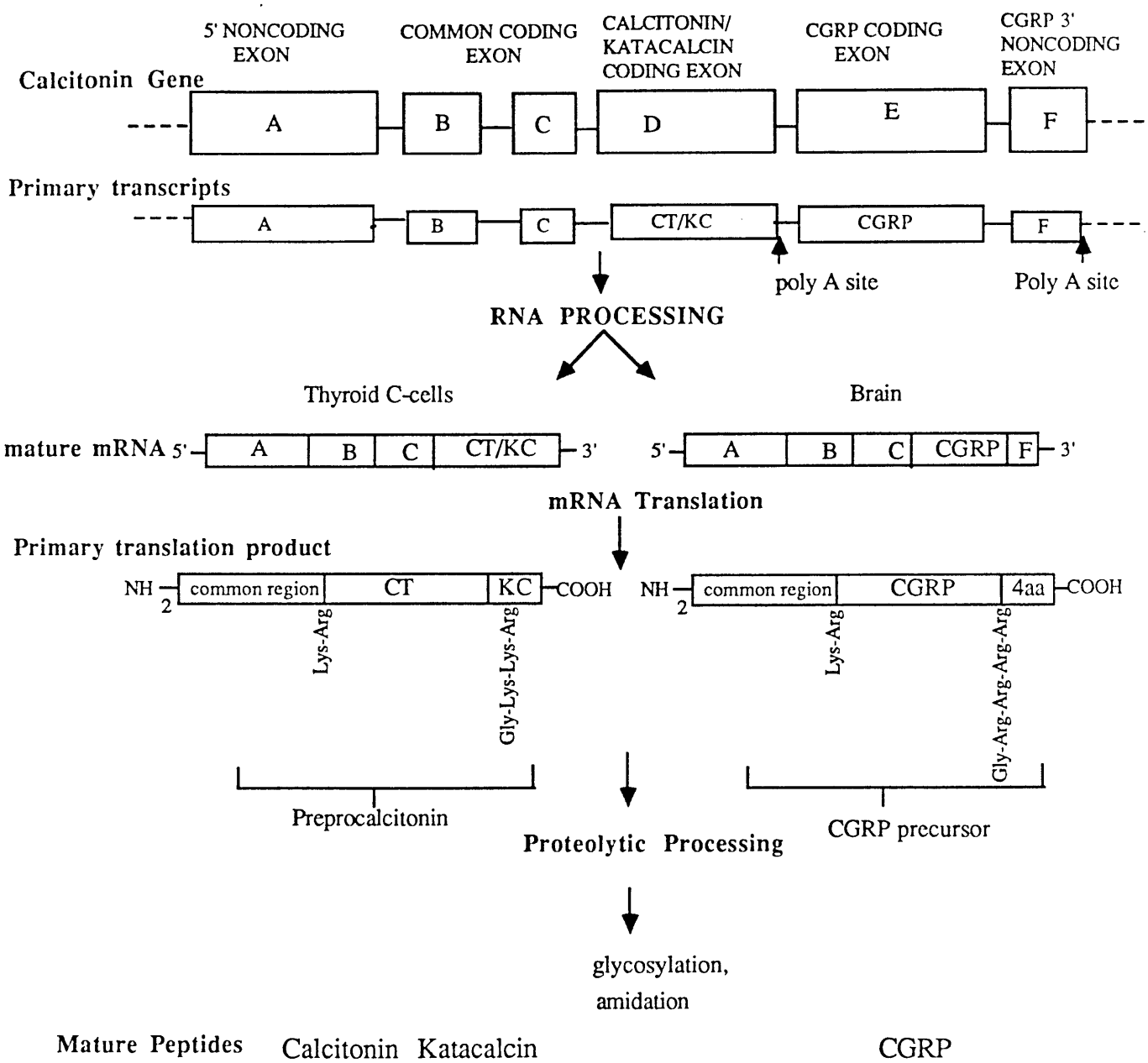


Figure 1: Schematic representation of biosynthesis of the peptides from the alpha-calcitonin gene

precursors to the peptide of interest in a cell-free system. A complementary DNA copy (cDNA) is then synthesised using viral reverse transcriptase. The cDNA is then inserted into a well characterised bacterial plasmid at a site in a gene coding for antibiotic resistance and the recombinant plasmid is introduced into living cells. After an initial selection, the bacterial cells are plated out on growth medium with and without the antibiotic in question, replicates of clones showing antibiotic resistance are then screened for the presence of coding sequences of interest by hybridisation to the initial mRNA or to synthetic oligonucleotide probes. Positive clones are cultured to amplify further inserted cDNA sequences before extraction and sequence analysis.

Examples of single-hormone precursors which have been sequenced by this method include rat insulin (Ullrich, Shine, Chirgwin, *et al.*, 1977), rat growth hormone (Seeburg, Shine, Martial, *et al.*, 1977) bovine parathyroid hormone (Kronenberg, McDevitt, Majzoub, *et al.*, 1979), and rat relaxin (Hudson, Haley, Cronk, Shine and Niall, 1981).

The nucleotide sequence of cloned cDNA coding for bovine pre-opiomelanocortin (POMC) (Nakanishi, Inoue, Kita, *et al.*, 1979) was the first to be reported for a polyprotein hormone precursor containing multiple bioactive peptides.This data was of great interest since in addition to confirming previously known amino acid sequences, it showed that pairs of lysine and arginine residues separated each of the component peptides, suggesting tryptic cleavage. Further example of polypeptide hormone precursors in which the component peptides were predicted to be separated by basic amino acid residues usually in pairs were corticotrophin releasing factor precursor (Furutani, Morimoto, Shibahara, *et al.*, 1983). Recombinant DNA technology has proved to be of great commercial significance for synthesis of peptides and proteins.

1.1.11.2. Nucleotide sequence analysis of calcitonin precursors.

Rat calcitonin precursor

A partial nucleotide sequence of cloned cDNA complementary to calcitonin mRNA from rat medullary thyroid was determined by Amara and co-workers in 1980 (Amara, Rosenfeld, Birnbaum and Roos, 1980). The nucleotide sequence encoded the expected amino acid sequence of rat calcitonin (Byfield, McLaughlin, Matthews and MacIntyre, 1976). Additionally, the existence of a new 16 amino acid peptide extending C-terminally from the rat calcitonin sequence to the C-terminal of the precursor was deduced.

The sequence of a cloned cDNA encoding the complete rat CT precursor was reported (Jacobs, Goodman, Chin, *et al.*, 1981). The C-terminal portion of the precursor was as predicted by Amara *et al.* (1980) with the sequence gly-lys-lys-arg separating CT from the C-terminal flanking peptide. The predicted N-terminal flanking peptide separated from CT by a pair of lysine and arginine residues, contained 82 amino acid residues, out of which 25 comprised the expected leader sequence (see figure 2).

Structure of human calcitonin precursor

The construction, identification and partial characterisation of two cDNA clones, which together were complementary to an estimated 60% of mRNA from medullary thyroid carcinoma and coded for the human CT precursor was described by Allison, Hall, MacIntyre and Craig (1981). The mRNA was about 1000 nucleotides long and directed the synthesis in a cell-free translation system of the precursor protein with a molecular weight of 21000.

The nucleotide sequences of these cloned cDNA's were reported by Craig, Hall, Edbrooke, Allison and MacIntyre (1982). The expected sequence of human CT was shown to reside towards the C-terminal end of its precursor protein. The comparison of the human and rat CT aminoacid sequences on the precursors indicated that there was less homology between the flanking peptides

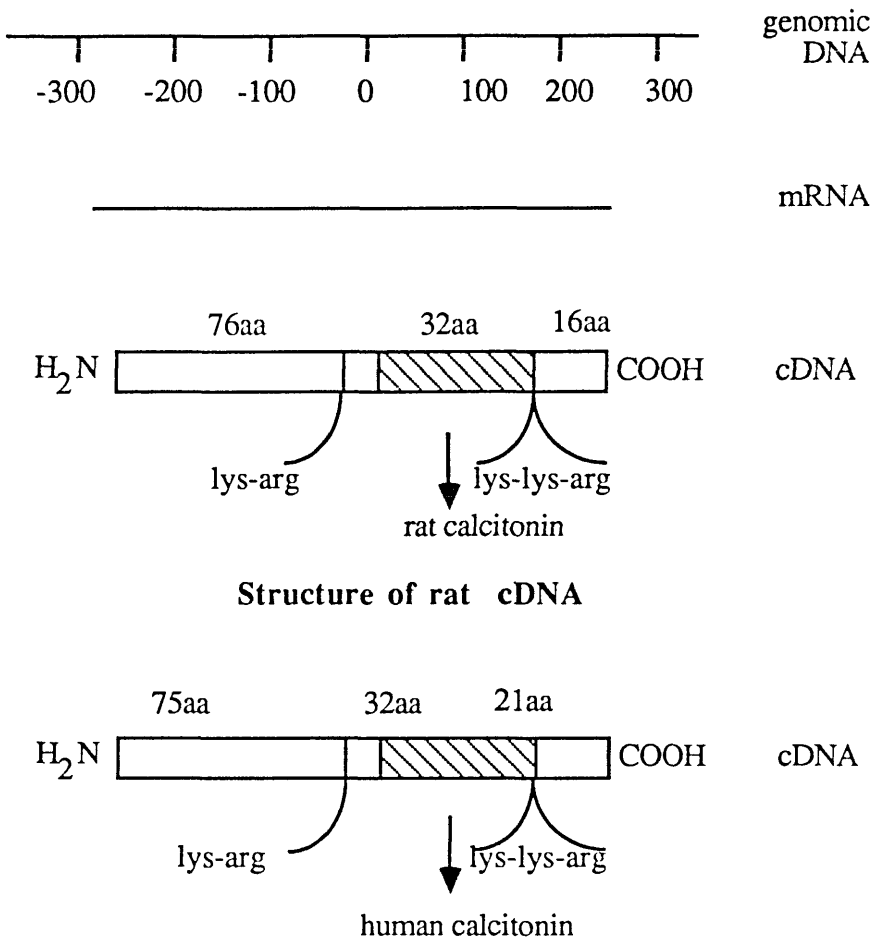


Figure 2: Comparison of cDNA structure in rat and man

than between the calcitonins. The human C-terminal peptide comprised 21 residues and was named Katacalcin whereas the corresponding rat C-terminal peptide was only 16 amino acid residues. The most striking feature of both the human and rat calcitonin N-terminal flanking peptide was the high content of acidic amino acid residues. There was also a very high proportion of hydrophobic residues in the known N-terminal portion of human peptide, in common with the corresponding rat sequence. In both precursors, the paired and triple basic residues separating calcitonin from the N- and C-terminal flanking peptides were conserved.

Another shared feature of the predicted human and rat precursor was the location of glycine residue next to the C-terminal proline of the CT sequence. A similar arrangement has been observed for other biologically active peptides which are synthesised as part of a larger precursor and subsequently amidated at the C-terminal during processing to the mature, secreted form (Cohn, Chang, Bowyer and Hellinh, 1973). The gly-lys-lys-arg sequence was also observed by Birnbaum, *et al.*, (1982) to be present in corticotrophin (ACTH).

A specific C-terminal amidating enzyme from the pituitary has been demonstrated with the ability to convert peptides that terminated in glycine to the corresponding peptide amide, using glycine as the amino donor (Bradbury, Finnie and Smyth, 1982). So it is likely that similar cleavage and amidation occurs during the processing of CT from its precursors.

Role of N- and C-terminal peptides in the precursor

The role of these peptides is unclear. They could serve for glycosylation to maintain the conformation of the precursor so that the specific enzymes required for maturation can act.

The N-terminal peptide is mainly hydrophobic and the main role of this peptide signal may be to permit the exportation of calcitonin after cellular secretion.

The role of the C-terminal peptide has been studied. In the rat, it was named

carboxyl cleavage peptide (CCP) by the group that identified it (Amara, Jonas, O'Neil, *et al.*, 1982). With the help of a radioimmunoassay utilising an antiserum raised in rabbits immunised with the prepared synthetic analogue of CCP coupled to keyhole limpet haemocyanin enabled the location of CCP in tissues producing CT.

In man, Craig, Hall, Edbrooke, Allison and MacIntyre (1982) identified, sequenced the C-terminal peptide and called it PDN-21 according to the chemical terminology of Tatemoto and Mutt (1981). Another name 'katacalcin', was adopted. The homology between katacalcin and rat peptide PDN-16 are in the N-terminal position rather than in the C-teminal.

1.2.Calcitonin gene-related peptide (CGRP): Polymorphism in the calcitonin gene expression

The production of multiple mRNA from the CT locus was initially observed in rat medullary carcinoma by Roos, Yoon, Frelinger, Pensky, Birnbaum and Lambert (1979). Spontaneous switching of serially transplanted rat MTC cultures changed from states of high to low CT production. Rosenfeld, Amara, Roos, Ong and Evans (1981) and Amara, Jonas, O'Neil *et al.*, (1982) showed that in CT producing tumors, the predominant 'total' and cytoplasmic polyadenylated RNA hybridising to cloned cDNA probes for the CT precursor was about 1050 bases in length. However, total polyadenylated RNA from low CT producing tumors also contained a large transcript of about 4000 bases as well as a major species of 1250 bases which hybridised to the probe. These authors considered the possibility that differential splicing of exons in heterogeneous nuclear RNA transcripts of CT was occurring in the two types of tumors.

The CT associated mRNA was found to be replaced by about 200 nucleotide long mRNA which was found to encode a protein of MW 16000 which did not contain immunoreactive CT. Genomic DNA and RNA analysis revealed that the rat calcitonin related mRNA encoded a 128 amino acid precursor polypeptide (Rosenfeld, Mermod, Amara, *et al.*, 1983). The latter molecule contained a 37 amino acid peptide, calcitonin gene-related peptide (CGRP) separated at its

carboxyl terminus from a tetrapeptide by a gly-arg-arg-arg sequence. At its amino terminus it was flanked by lys-arg residues separating it from a larger peptide which was found to be homologous with the amino terminal peptide of the CT precursor.

The point of diversion, suggestive of a splicing site occurred C-terminally to the serine residue. Polyadenylated mRNA from rat hypothalamus were found to hybridise to both CT and CGRP cDNA probes but were the same size as the CGRP encoding mRNA species present in low CT producing tumors. Amara, *et al.*, (1982) postulated that tissue-specific expression of the CT gene achieved by differential splicing of primary transcript mRNA might occur for the predominance of CGRP mRNA over CT mRNA observed in the hypothalamus.

So, the CGRP sequence was found to be contained in the fifth exon. The sixth exon is also a part of the CGRP transcript. It is not translated and ends by the alternative polyadenylation signal ATTAAA (Jung, Sippel, Grez and Schultz, 1980). Thus both CT and CGRP mRNA's contain identical 5' non-coding and coding regions.

In man, the first 225 nucleotides of coding information (75 amino acids) are identical in both calcitonin and CGRP mRNAs (Craig, Hall, Edbrooke, Allison and MacIntyre, 1982). This region differs by only 44 nucleotides (20 amino acids) from the corresponding rat sequence (Amara, *et al.*, 1982). The sequences of calcitonin and CGRP mRNAs then diverge entirely. As with calcitonin, the CGRP coding region is better conserved than other regions (Lasmoles, Jullienne, Day, *et al.*, 1985; LeMoullec, Jullienne, Chenais, *et al.*, 1984).

1.2.1. Isolation of CGRP

CGRP was first isolated from human medullary thyroid carcinoma tissue and sequenced by fast atom bombardment mass spectrometry (Morris, Panico, Etienne, Tippins, Girgis and MacIntyre, 1984) and provided the rigorous evidence for the actual existence of the predicted peptide and also established its presence in man.

Human α-CGRP has been found to differ from the predicted rat α–sequence

by four amino acids. Human β-CGRP has been found to differ from the α form by three amino acids. There is about 30% structural homology between the structure of the CGRP peptide and salmon CT (for review see Zaidi, Breimer and MacIntyre, 1987).

1.2.2. Biosynthesis of CGRP

From a single transcription unit, multiple mRNAs can be generated (Leff, Rosenfeld and Evans, 1986). It was proposed by Amara, *et al.* (1982) that calcitonin and CGRP mRNAs are generated by different splicing of a transcript of a whole gene (see figure 1). The synthesis of CT and CGRP mRNAs is believed to start from a common site. Transcription proceeds in both the calcitonin and CGRP exon at a similar rate and terminates 1 kilobase downstream of the sixth exon. Polyadenylation precedes and determines the choice of splicing. Alternative processing has been confirmed but it has been reported that polyadenylation at the calcitonin site generally follows the removal of introns (Bovenberg, Van de Meerendonk, Baas, *et al.*,1986).

Alternative splicing has been reported *in vivo* and *in vitro* due to RNA secondary structure (Solnick,1985). Differential splicing of exons has also been demonstrated in the case of the insulin-like growth factor I gene (Rothwein, Pollock, Didier and Krivi, 1986).

Recently, another CT gene (β gene) has been identified in rat and man (Hoppener, Steenberg, Zandberg, *et al.*, 1985; Jonas, Lin, Kawashima,*et al.*, 1985; Alevizaki, Shiraishi, Rassool, Ferrier, MacIntyre and Legon, 1986). This gene is also localised to the short arm of chromosome 11. It appears to have arisen due to local duplication of the α-gene. Its mRNA organisation has been found to be similar to that of α–CGRP. However, the 5' and 3' non-coding regions of the gene have been found to diverge significantly. This has permitted the generation of cDNA probes specific for the two genes. In rat and man, the α and β sequences have been found to differ by one and three amino acids respectively. To date there has been no evidence for the production of the CT-

associated mRNA from this gene. Alevizaki, *et al.* (1986) have cloned the CT-like sequence (exon 4 -like) region of the human β gene.

1.2.3. Localisation

The presence of CGRP mRNA in the thyroid (Sabate, Stolarsky, Polak, *et al.*, 1985) as well as the presence of immunoreactive CGRP in the normal rat and human thyroid has been reported by several groups (Tschopp, Tobler and Fischer, 1984; Self, Wimalawansa, Johannsson, *et al.*, 1985). In man, the CGRP content in the thyroid is less than that found in the pituitary, whereas in the rat, it is reversed.

In the C-cells, CGRP was found to be present in normal and malignant conditions (Nitta, Kito, Kubota, *et al.*, 1986). It was predominantly localised with calcitonin in the same granules. However, there is also a significant population of CGRP positive cells that have been found to appear on the 17th day of gestation, and prior to birth, are mainly localised to the centre of the thyroid.

CGRP immunoreactivity has been demonstrated extensively in the central nervous system. A number of studies have shown that CGRP often co-exists with substance-P. Other studies have confirmed that most substance-P immunoreactive cells which are small show the presence of CGRP immunoreactivity whereas only a proportion of CGRP- immunoreactive cells which are bigger are substance-P positive (for review see Zaidi, Breimer and MacIntyre, 1987).

Apart from the central nervous system, CGRP has been found to be distributed in the peripheral nervous system, throughout the digestive system, in the cardiovascular system,and the respiratory tract in several mammalian species, and in the medullary thyroid and lung carcinomas.

1.2.4. Biological Actions of CGRP

1.2.4.1. Action on the bone

CGRP has a weak CT-like effect in the rat. It causes hypocalcaemia at 100-1000 fold higher doses than those of calcitonin (Tippins, Morris, Panico *et al.*, 1984; Roos *et al.*, 1986). It also stimulates cyclic AMP formation in mouse calvaria at similar concentrations (Bevis, Zanelli, Lane, Morris and MacIntyre, 1986). It inhibits both basal and stimulated resorption of intact bone (D'Souza *et al.*, 1986) as well as spreading, motility and resorption by isolated osteoclasts (Zaidi, *et al.*, 1986)

The effect of CGRP *in vivo* in the rabbit is that of hypocalcaemia at doses comparable with those of CT. However, at higher doses there is a sustained PTH-like effect at all dose levels (Zaidi, *et al.*, 1987)

1.2.4.2.Action on central nervous system (CNS).

CGRP produces marked effects on physiological, behavioural and metabolic functions mediated by the CNS and the basic mechanism of these effects appears to be modulation of neuronal excitability (Twery and Moss, 1985). CGRP administered centrally causes suppression of feeding. This effect was similar to, though less marked than that of calcitonin, cholecystokinin or bombesin. Other effects on the CNS have been reviewed by Zaidi, *et al.*, 1987.

1.2.4.3. Peripheral neural effects

It has been postulated that CGRP may be a sensory neurotransmitter and this is evident from its distribution, co-localisation with tachykinins and its depletion following treatment with capsaicin. On the basis of its occurrence in the autonomic ganglia and the CGRP rich innervation of cardiovascular, gastro-intestinal and special sensory system, it has been suggested that it may have a role as a neurotransmitter.

1.2.4.4. Actions on cardio-vascular system

Intra-cerebroventrically administered CGRP has been found to stimulate noradrenergic outflow selectively accompanied by intense tachycardia and hypertension. Microinjection of CGRP into central amygdala nucleus has been found to produce similar effects on heart rate and blood pressure (Nguyern, Sills and Lacobowitz, 1986). When injected intravenously, CGRP produces hypotension and tachycardia, in both experimental animals and man (Fischer and Born, 1985; Tippins, Marzo, Panico, Morris and MacIntyre, 1986; Struthers, Brown, Beacham, Morris and MacIntyre, 1986). In man, CGRP concentration as low as 56 pmol/l caused flushing and an increased level of both catecholamines.

The hypotensive effect of CGRP is due to its direct effect on blood vessels (Brain, Williams, Tippins, Morris and MacIntyre, 1985). The demonstration of such an effect has been the first recognition of the vascular actions of CGRP. Intra-dermal injections in rabbit skin of CGRP in the range of femtomolar concentrations have been found to induce intense microvascular dilatation with increased blood flow. The arterioles alone of hamster cheek pouch preparations have been found to be markedly dilated following a topical application of CGRP.

In human volunteers intradermal injection of CGRP produced reddening or a permanent flare reaction which lasted twelve hours. This CGRP-induced vasodilation is a direct effect of the peptide and is not due to the release of classical neurotransmitters.

Human α CGRP has been found to be similar in potency to human β CGRP, and PGE2 and approximately 10-1000 fold more potent than acetyl choline, ATP, ADP, adenosin,5-HT, substance-P, isoprenalin or VIP (Brain, Williams and MacIntyre, 1986).

CGRP has been shown to cause vasodilation of the coronary vasculature. In man, a dramatic decline of perfusion pressure has been coupled with a clear-cut radiographic demonstration of dilated coronary arteries (McEwan, Larkin, Davis, *et al.*,1986). Similar effects have been observed in isolated rat, rabbit and pig heart preparations (Holman, Craig and Marshall, 1986).

Studies by Brain *et al.*(1985) and Kubota, Moseley, Butera, *et al.* (1985) have shown that CGRP induced vasodilation is endothelial tissue dependent in at least major veins. The role of endothelial derived relaxing factor is unclear. The hypotensive effect of CGRP has been mimicked by capsaicin and it has been shown that CGRP is released from the perivascular and cardiac nerves in response to stimuli (Zaidi *et al.*, 1985). It is most likely that the release of peptide acts on smooth muscle receptors and then spills over into the circulation. It is unclear whether CGRP is merely a vasodilator neurotransmitter or has a hormonal role in the physiological regulation of blood flow.

1.2.5. Plasma CGRP

1.2.5.1. Normal subjects

Normal circulating levels of human α-CGRP in man were investigated by Girgis, Macdonald, Stevenson, *et al.* (1985). They developed a sensitive and specific RIA to measure plasma levels in 51 normal, healthy subjects. Immunoreactive CGRP was detected in the plasma of 50 subjects. Levels ranged from 9.7 to 71 pmol/l. Unlike hCT, there was no significant difference between the mean level in males and females. There was also no correlation with age in either sex.

The mean plasma hCGRP level was five times higher than that of hCT and no correlation between plasma hCT and hCGRP level was observed.

1.2.5.2. Levels in MTC

In a study carried out on the levels of plasma CT and CGRP in 35 patients with MTC, CGRP levels were elevated in 26 patients. CT levels were found to be higher than those of CGRP, although the ratio of the two peptides varied from patient to patient. These results also suggested that absolute values of either CT or CGRP in plasma have no direct relationship to aggressiveness of disease. Plasma CGRP appeared to be a useful additional marker for the disease. In another study of 45 patients with MTC, plasma CGRP was elevated in approximately 50% of cases (Girgis, Lynch, Hillyard *et al.*, 1987). There was a weak correlation between CGRP and CT levels. They concluded that CGRP was not a sensitive marker for MTC and that the measurement of hCT or KC is

superior for the detection of MTC.

1.3. Katacalcin

A second peptide, Katacalcin (KC, PDN-21) which flanks CT within the human CT precursor was discovered by the application of recombinant DNA technology just like CGRP. The structure of KC has been fully confirmed by formal isolation of the peptide from MTC and structural determination by mass spectroscopy (Arnett, 1984). Immunohistochemical studies have enabled the co-localisation of the peptide with calcitonin in the C-cells in normal thyroid and in thyroid tumors (Ali-Rachedi, Varndell, Facer, *et al.*, 1983). A synthetic tyrosine containing analogue of KC was used to establish an RIA in order to measure circulating levels of the peptide. Preliminary data (MacIntyre, Hillyard, Murphy, *et al.*, 1982) indicated that immunoreactive KC circulated in approximately equimolar concentrations in patients with MTC.

1.3.1. Plasma levels

1.3.1.1.Normal subjects

An RIA has been developed for KC (MacIntyre *et.al.*, 1982) by raising antibodies in rabbits by injecting tyrosinated KC coupled to ovalbumin. The serum was used at a dilution of 1:1200 with ^{125}I-tyrosinated KC as tracer. There was no cross-reaction with up to 10μg/tube of LHRH, sCT, hCT, somatostatin, insulin and many other peptides. The limit of sensitivity was 40 pg/tube.

In 15/16 patients with MTC, the KC level was elevated. The concentration ranged from 0.6-138 ng/ml and was found to be approximately equimolar in concentration with CT levels (0.8-314 ng/ml). The KC levels were not measurable in normal subjects at this sensitivity.

By prior extraction of the peptide from plasma, normal circulating levels were measured in 57 normal volunteers. As with hCT, plasma KC levels were higher in men than in women.

1.3.1.2. Plasma levels in patients with MTC

Earlier findings that KC circulates in high concentrations in MTC patients were confirmed by several groups (Girgis, *et al.*, 1987 and Roos, Huber, Birnbaum, *et al.*, 1983) and circulates in equimolar concentrations with CT. Furthermore, KC release responds to the same stimuli as CT. Therefore, diagnostically, KC is as good a marker as CT for the detection of MTC and C-cell hyperplasia.

Secretion of KC and CT has also been described in MTC tissue explants as well as cultures of MTC cultivated *in vitro* over a period of 10 to 50 days, whilst continuously secreting peptides of the CT family (Raue, Serve, Rix and Zeigler, 1987). This release of KC and CT has also been described in perfused human MTC (Iwasaki, Myers and Freake, 1983).

1.4. Amylin

Amylin is a 37 amino acid polypeptide first isolated as an islet amyloid from the cat pancreas. It has 46% homology with CGRP (β) (for details see MacIntyre, 1989).

MacIntyre, I.(1989). Amylinamide, bone conservation and pancreatic β cells. *The Lancet* **ii**, 1026-1027.

Section II

Chapter 2: Development of non-isotopic immunoassays for human CT and rat CGRP using polyclonal antisera

Summary

Colorimetric enzyme immunoassays (EIA's) were developed for hCT and rat CGRP using enzyme amplification. This involved the dephosphorylation of NADP, a substrate for alkaline phoshatase to NAD which then activated a highly NAD-specific redox cycle to produce an intense colour. This technique enhanced the sensitivity of the resulting assays.

The assay for hCT was directly comparable with the conventional RIA except that a microtitre format was used to facilitate separation of the antibody-bound conjugate from the unbound material. The sensitivity of the EIA (6 pmol/l) was at least three times higher than that of RIA (20 pmol/l). There was good correlation (r=0.96) between the two methods.

For rat CGRP, an enzyme immunometric assay was developed using two polyclonal antisera already at hand. One antiserum had been raised against the whole molecule (1-37 amino acid) alpha hCGRP, but was particularly reactive to the mid-molecule region, while the second antibody had been raised against a C-terminal decapeptide fragment of the rat CGRP. The resulting assay was not only superior in terms of sensitivity, but was also more specific than the RIA. The sensitivity of the overnight EIA was comparable to the 5- day RIA. The assay was successfully applied to investigate plasma levels of rat CGRP.

This study is an illustration of the possible development of a two-site assay for small peptides using polyclonal antibodies.

Chapter 2: Development of a non-isotopic immunoassay for hCT and rCGRP using polyclonal antisera

2.1. Introduction

In the late 1950's, two independent papers first described an immunoassay technique suitable for the measurement of important hormones. Yalow and Berson (1959) described the first radioimmunoassay (RIA) for insulin whereas Ekins (1960) described a method for the determination of serum thyroxine using a competitive binding assay. In the latter test system, the naturally occurring thyroxine binding globulin was used as the binding protein and radio-labelled thyroxine was used as the tracer. Today, there is a vast array of immunoassay methods available for the measurement of a wide range of analytes present in biological fluid.

The RIA consists of four components, namely the specific anti-analyte antibody, the analyte and the radiolabelled analyte and a suitable separation system. The assay depends on competition between the radiolabelled analyte and the non-labelled analyte in the sample or standard for a limited number of binding sites on the specific antibodies. The reaction follows the law of mass action and the labelled and non-labelled analytes will bind to the antibody in proportion to their relative concentrations in the reaction mixture. The degree of binding of labelled analyte to antibody is inversely related to the concentration of unlabelled analyte in the test medium. After a suitable separation step to separate antibody-bound analyte from the free form, quantification of the analyte present in the sample is achieved by comparing the level of inhibition of radio-active signal with that observed for known standard of the analyte.

High degrees of sensitivity and specificity could be achieved with immunoassays and for this reason, they have rapidly replaced many other analytical methods used to detect biologically important substances. Radioisotopes have been used most extensively for labelling of analytes and RIA is still the method of choice for large scale operation.

Maximal sensitivity of a RIA is achieved when using a minimum amount of antibody (Ekins, 1985) and so, assays for analytes present in low concentrations, such as calcitonin in normal subjects, require lengthy incubation and counting times since the antibody concentration is low (that gives 30% specific binding). This is one of the major disadvantages of RIA.

Radioactive isotopic labels have a limited shelf life and quantum yield (signal per unit time). In addition, there are legal restrictions, such as the use and disposal of such isotopes, resulting in the assays being limited to certain centres only.

These considerations have led workers to search for alternative labels for antibodies and analytes. Many candidates have been proposed; for example, fluorescent labels (Cukor, Woehler, Pensiani and Ferim, 1976), metals (Cais, Dani, Eden *et al.*, 1977) and free radicals (Leute, Ullman, Goldstein and Herzenberg, 1972) but at present, the most promising new labels are enzymes.

Enzymes can be linked to antibodies or analytes such that the complexes retain both immunological and enzyme activity. Degradation of a chromogenic substrate by the enzyme enables accurate and sensitive detection of the presence of the enzyme-linked component. Figure 3 outlines the various types of protein binding assays.

2.1.1. Classification of Enzyme immunoassay

Enzyme immunoassays (EIA) are classified into two main groups, namely homogeneous and heterogeneous, on the basis of whether the assay requires a separation step or not.

2.1.1.1. Homogeneous EIA

In this system, the analyte is labelled with an enzyme such that the enzyme retains its activity. When this enzyme-labelled analyte reacts with an antibody, the enzyme activity of the conjugate is altered (usually inhibited). In the test, the unknown sample is mixed with the conjugated analyte and its specific antibody. If there is analyte in the sample, it competes with the conjugated analyte for a

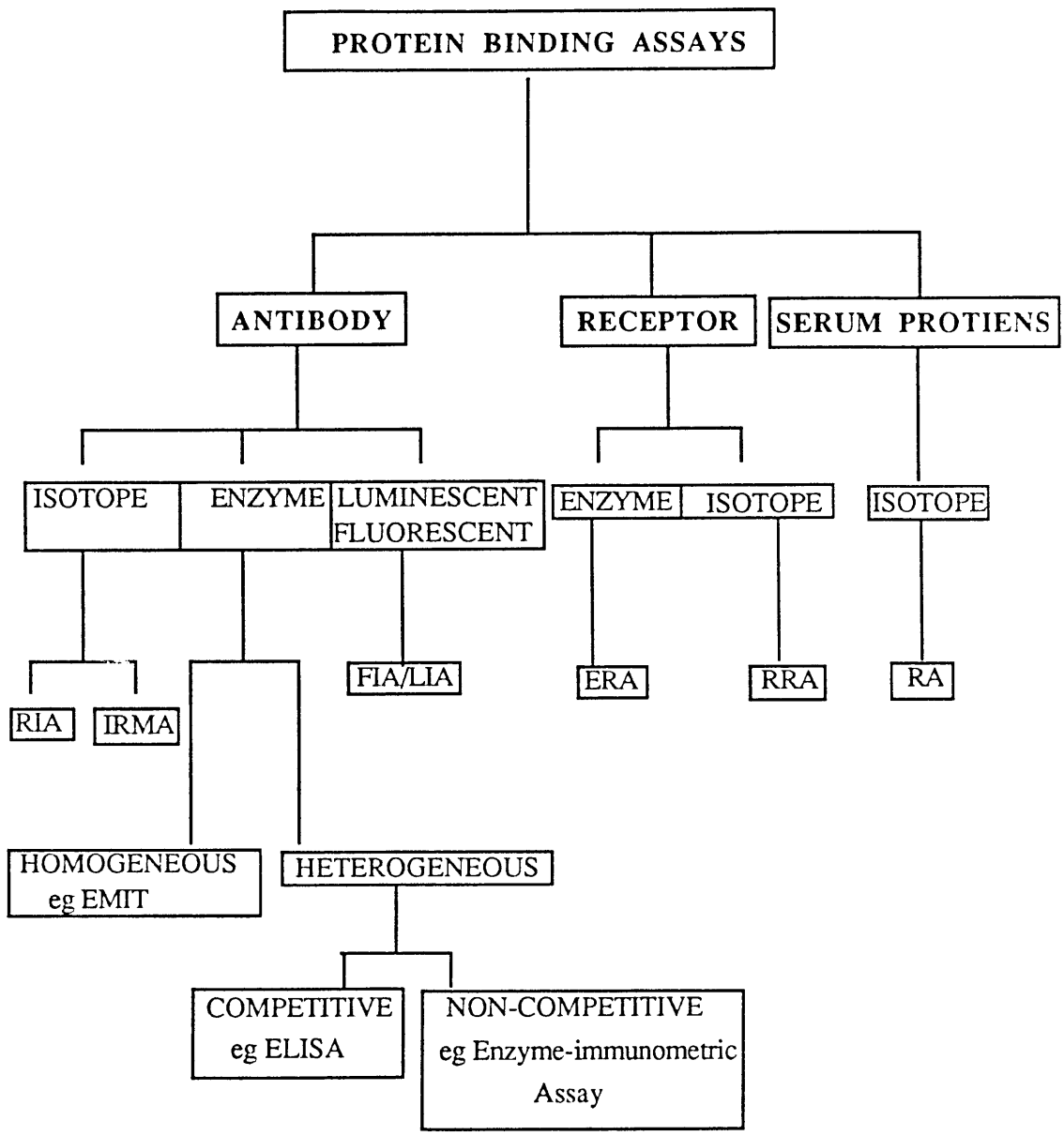


Figure 3: General Classification Of Protein Binding Assays

limited number of antibody binding sites. Thus there is less antibody available to inhibit the enzyme activity of the conjugate, and upon addition of substrate, there is a positive signal. This method forms the basis of the enzyme multiplied immunoassay technique (EMIT system[1]). This system is useful for rapid assay of low molecular weight compounds such as drugs and steroids (Galan, Forman, O'Brien and Brown, 1976). It is remarkable that in this system, two different reactions take place simultaneously in the reaction vessel. No separation of bound and free analyte is required and therefore the manipulations are reduced to a minimum.

The main disadvantages of these homogeneous assays are that firstly they are not as sensitive as heterogeneous assays and secondly, as they are based on steric inhibition of enzymes, they cannot be used generally to assay large molecules such as peptides and proteins. However, more recently an EMIT-type of assay for IgG has been reported by Gibbons, Skold, Rowley and Ullman (1980).

2.1.1.2. Heterogeneous EIA

Heterogeneous EIA's are so called because of the separation of bound and free analyte is involved. These assays are further classified into two main groups, competitive and non-competitive immunoassays.

The competitive EIA is analogous to conventional RIA. The only difference is in the use of an enzyme rather than an isotope as the tracer (for review see Voller, Bartlett and Bidwell, 1978). Scientifically, this may seem to be a small difference but it can result in quite a marked difference in the development of an assay.

In this system, the analyte is labelled with an enzyme and mixed with the test sample or standard containing the analyte which then competes for a limited number of antibody binding sites. The bound analyte is separated from the free form and its enzyme activity estimated by addition of a substrate.

The non-competitive assay first described by Miles and Hales (1968) made

[1]Syva corporation, USA.

use of isotope-labelled antibodies in a non-competitive radioimmunoassay and indicated that enzymes could be used as labels.

In this type of assay, the analyte in the sample or standard is first captured by the excess antibody which is coated on to the solid phase. Another enzyme-labelled antibody is then added which binds to the captured analyte. This assay format is also known as a "sandwich" assay or two-site immunometric assay as it requires the analyte to have at least two reactive antigenic sites (epitopes) and therefore is suitable only for large molecules. It also means that the assay can be formulated as a "one-step" procedure where the analyte and the labelled antibody are placed on to the antibody-coated solid phase, thereby increasing the resulting specificity and improving speed and convenience. Nearly all the tests are in the micro-plate format which permits easy testing of large number of samples.

There has been some delay in the wide use of immunometric assays. The reason for this has been the unavailability of labelled antibody, since polyclonal antisera were the only available source of antibody and these required immunoaffinity chromatographic purification. The latter is a long procedure which gives a limited yield and suffers from the inconvenience that the antibodies with high affinity are precisely the ones which are most difficult to elute.

The availability of monoclonal antibodies has assured an essentially unlimited source of specific material for either labelling or solid-phase preparation and hence wider application of immunometric assays.

2.2. Measurement of the peptides

2.2.1. Immunochemical heterogeneity of circulating peptides

The existence of multiple hormonal forms has been documented for several peptide hormones and this has affected their quantification. It was shown that such size and immunochemical heterogeneity also applies to hCT and CGRP because when plasma from patients with MTC was subjected to gel-filtration chromatography, several peaks reacting with anti-human CT were obtained. (Singer and Habener, 1974; Sizemore, Heath and Larson,1975; Deftos, Roos, Bronzert and Parthemore, 1975; Babe, Esbrit and Rapido, 1985). The precise nature of these peaks is not fully known but they are thought to be possibly variations in biosynthesis, metabolism, polymerisation and/or non-covalent binding to other proteins.

In vitro studies by Roos, Okano and Deftos (1974) using preparations of both human and trout calcitonin producing cells showed that the presence of a biosynthetic precursor to CT exists. Later investigations using cell-free translations of polyadenylated CT mRNA, followed by non-denaturing polyacrylamide gel-electrophoreisis (PAGE), by Van Der Donk, Van Dam, Goudswaard, Hackeng and Lips (1976) and Lips, Van der Sluys Neer, Van der Donk, Van Dam and Hackeng (1978) confirmed the presence of a common translation product with MW 40,000. Other studies carried out using the same system but analysing the product on denaturing SDS-PAGE methods confirmed the presence of primary immunoprecipitable products with MW 11,000 to 21,000, suggesting cleavage of pre-procalcitonin to procalcitonin.

Studies by Jacobs, Goodman, Chin, *et al.* (1981) showed that translation of mRNA from rat MTC tissue yielded a product with MW 17,000. As this product bound to concanavalin-A sepharose and could be cleaved to a 12,000 MW product by an enzyme of the endoglycoside group, they concluded that the 17,000 MW product CT precursor was glycosylated procalcitonin and that the potential site of glycosylation was the asparagine residue at position 3 on the CT sequence. Up to 5-20% glycosylated immunoreactive CT has also been detected

from human MTC and small cell carcinoma of the lung (Baylin, Weiman, O'Neil and Roos, 1981). In extracts of serially transplanted rat MTC, glycosylated CT with MW up to 27,000 has been detected (O'Neil, Birnbaum, Jacobson and Roos, 1981).

In normal human plasma, immunoreactive heterogeneity of CT was also observed (Girgis, 1980). One peak had the same elution position as synthetic monomeric CT, three peaks had apparently higher molecular size than the CT-monomer while two small peaks (probably representing the fragments) eluted later than CT-monomer. Polyclonal antisera were used to screen the column fractions.

In a recent study, it was reported that after gel-filtration of peripheral blood from one MTC patient and using a monoclonal based IRMA to detect the immunochemical forms of CT, two major peaks were detected. One eluted in the void volume and the other eluted in the same position as ^{125}I-hCT (Motte, Ait-Abdellah, Alberici, *et al.* 1987). In a patient with liver metastasis of MTC, several immunochemical peaks were detected which after further treatment with 6M guanidine and dithiothreitol resolved into one monomeric peak. They concluded that in the latter case, it was the presence of polymeric forms of hCT which were detected by the IRMA whereas in the former case, only the monomeric and the protein bound hCT was detected.

Immunochemical heterogeneity of the peptides in plasma affect their measurement to a significant extent so when developing new assays, efforts are made to improve the specificity of the quantification methods.

2.2.2. Bioassays

Plasma hCT levels in patients with MTC were first measured by *in vivo* bioassay which involved observing the hypocalcaemic effect in the young rat (Kumar *et al.*, 1963). However, due to the presence of low levels of CT in normals, large volumes of plasma were required for peptide extraction.

An *in vivo* second messenger bioassay has been developed for hCT based on the injection of calcitonin into neonatal rats and measurment of cAMP

released into the circulation. This assay is not specific for hCT and is at least ten fold less sensitive than the rat bioassay (20-100 IU) based on calcium release.

Organ culture bioassays are more sensitive with a detection limit of approximately 10pg/ml. These assays are based on the ability of calcitonin to inhibit the release of labelled calcium from neonatal long bones or calvariae (Chambers *et al.*, 1985).

Recently, an ultrasensitive and specific bioassay based on the inhibitory effects of CT on osteoclast motility and function has been sucessfully developed (Zaidi *et al.*, 1987). The sensitivity of the osteoclast bone resorption bioassay (0.1IU/ml or 3 atomol/ml) is about 30 to 40 fold higher than the whole animal bioassay. This assay has been shown to be highly specific for CT except that CGRP has a CT-like effect at 1000-fold higher concentration than that of CT. These assays were used in the present study to measure the biological activity of the immunochemical forms isolated from MTC plasma (chapter 4).

2.2.3. Immunoassays

2.2.3.1. Conventional RIA

The principles of the RIA are summarised on page 60. It was through the RIA approach that the value of measurement of plasma CT in the diagnosis and management of patients with MTC was well established. Apart from MTC, non-thyroidal malignancies have also been associated with elevated hCT (Coombes, Hillyard, Greenberg and MacIntyre, 1974). Circulating levels of these peptides have been established using RIA's.

Although RIA was a useful technique, problems existed because hCT has been shown to circulate in several distinct molecular forms. The circulating heterogeneous species of the peptide are uncharacterised and the binding of the antisera with each of the species is incompletely known, hence the variation in the measured values of plasma hCT between laboratories. Long incubations are required to achieve the desired sensitivity in order to measure the low levels of peptides in normal plasma.

2.2.3.2. Immunometric assays

The problem of sensitivity can be partly overcome by employing a tracer which can be detected by a highly sensitive detection system (for example, the enzyme amplification system used in conjunction with alkaline phosphatase-labelled tracer; see chapter 2). But in order to improve the specificity, immunometric assays (two-site) are necessary. An immunoradiometric assay for hCT has already been reported by Motte, Alberici, Ait-Abdellah and Bellet (1987) which employs specific monoclonal antibodies. A monoclonal-antibody based enzyme-immunoassay for hCT has also been reported by Berger, Shanes, Hart, Dissinger, Hill and Murayama, 1986).

2.2.3.3. Radio-receptor Assay

Although immuno-assisted methods are usually more sensitive than bioassays, an assay based on specific receptors rather than an antibody has the great advantage that it can reflect the biological activity which follows specific receptor binding.

A recently developed radio-receptor assay (RRA) for hCGRP is a competitive radioligand assay which measures displacement from a specific receptor-bound tracer hormone by unlabelled hormone in the standard or sample (Wimalawansa, 1987, unpublished communication). The RRA described in the above study utilises rat or human cerebellar membrane preparations as the source of receptor and ^{125}I-hCGRP as the radioligand for the receptor-reactive CGRP. The assay was found to be rapid (requiring 2 to 3 hours), robust, specific and comparable to a bioassay but with greater sensitivity.

An RRA for hCT has also been developed (Becker, Bivins, Radfar, *et al.*, 1978) using calvariae of newborn rats. More recently, CT receptors have been characterised for the first time on isolated rat osteoclasts (Nicholson, Moseley, Sexton, Mendelsson and Martin, 1986). No receptor-based assay for KC has been reported to date.

Tables 3 and 4 show the summary of the immunoassay sensitivities to date.

Assay	Year	Type	Incubation time	Detection limit	Other details
Clark et al.	1969	RIA	O/N	34 pmol/l	Extraction
Hennesey et al.	1974	RIA	5 days	8 pmol/l	plasma
Hillyard et al.	1977	RIA	5 days	3 pmol/l	Extraction
Becker et al.	1978	RRA	O/N	15 pmol/l	plasma
Girgis et al.	1987	RIA	7 days	6 pmol/l	plasma
Motte et al.	1987	IRMA	O/N	3 pmol/l	serum

Table 3: Summary of the hCT assay sensitivities published to date

CGRP assay	Year	Type	Incubation time	Detection limit	Other details
Girgis et al.	1985	RIA	7 Days	5-10 pmol/l	Plasma (human)
Zaidi et al.	1986	RIA	5 days	2.5 pmol/l	Extraction (Rat plasma)
Wimalawansa (unpublished observations)	1988	RRA	2 hr	40 pmol/l	Plasma (human)

Table 4: Summary of the CGRP assay sensitivities published to date

2.3. Non-isotopic immunoassays for hCT and CGRP

2.3.1. Competitive (one-site) assay for hCT using polyclonal antibodies

The first report of the development of a non-isotopic CT immunoassay was for frog calcitonin (fCT) (Robertson, 1986). A method of competitive inhibition enzyme-linked immunosorbant assay (ELISA) was used in this study. Synthetic salmon calcitonin (sCT) was initially bound to the solid phase of a microtitre plate. In a separate glass tube, the antibody against sCT raised in a rabbit was pre-incubated with fCT under non-equilibrium conditions. Subsequently, the coated plates were washed and the incubation mixture was added to the wells. Under these conditions, the unreacted antibody bound to the solid phase sCT with proportionally less antibody reacting with the solid phase with increasing amounts of soluble fCT. Following this incubation, the unbound reactants were washed from the wells and the plates re-incubated with goat anti-rabbit antibody conjugated to an enzyme horseradish peroxidase (EC 1.11.1.7.). The enzyme conjugate reacts with the available solid-phase antibody-antigen complex (see figure 4). After incubation, the plates were washed and incubated with a substrate. The amount of colour developed was inversely proportional to the amount of soluble fCT originally present in the initial antibody-antigen incubation. This method was found to have a sensitivity of 100pg/ml. The above format was impractical for hCT because of the number of incubation steps involved.

An enzyme immunoassay for elcatonin, a synthetic analogue of eel CT (eCT) has been developed by Aikawa, Ohayama, Mori, Hironaka, Miyaka and Morishita (1985). In this assay, elcatonin was conjugated to β-galactosidase and the product was used as the tracer. The assay sensitivity was found to be about 200 pg/ml in plasma. This was used to measure plasma levels of elcatonin after intramuscular or intravenous injection.

A more suitable assay format would be directly labelling the analyte with an

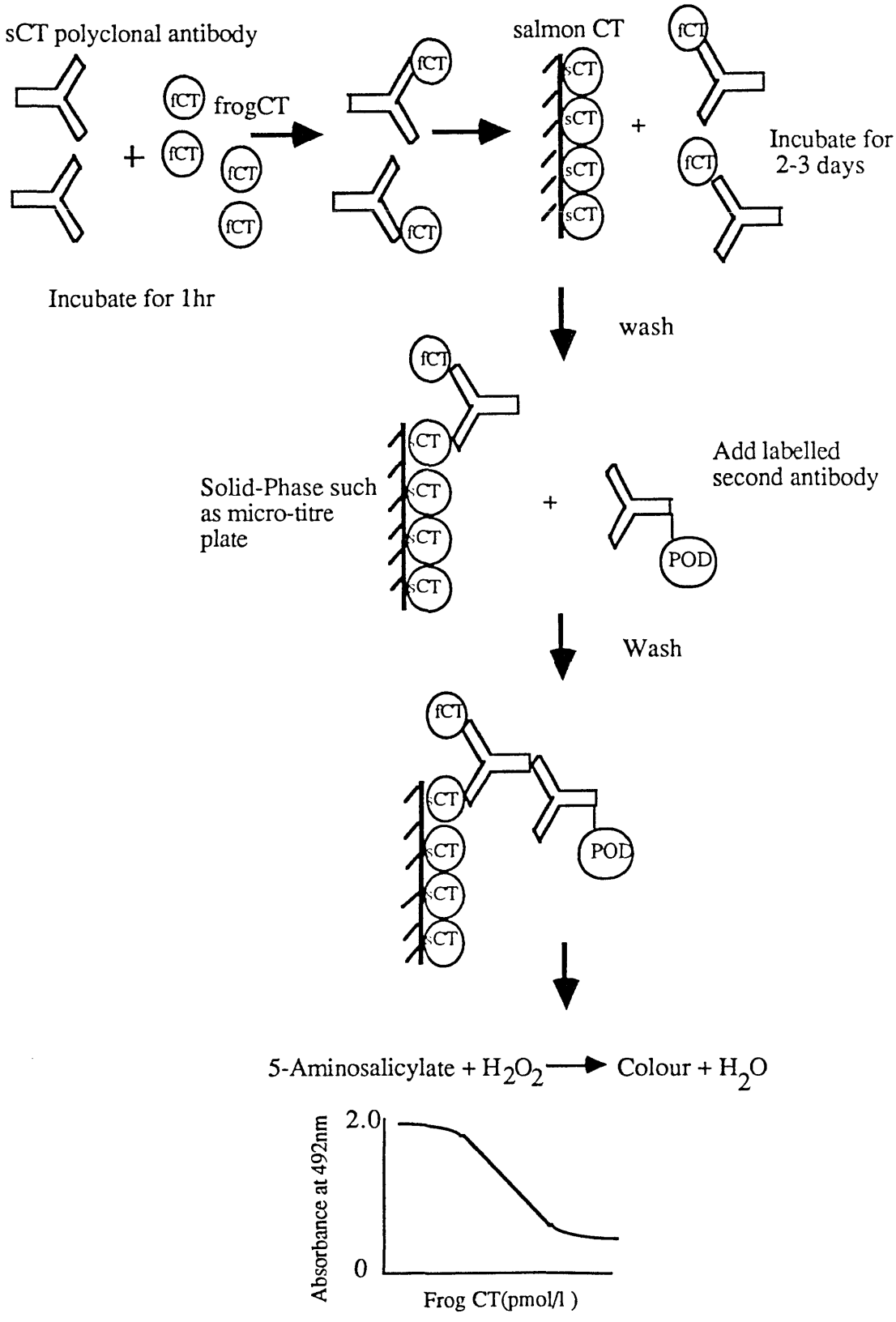


Figure 4: An example of a heterologous ELISA : an assay for frog calcitonin

enzyme and binding the antibody to the solid phase. This would allow direct comparison of the EIA with the RIA using the same antibody. This format (see figure 5) was adopted for the one-site calcitonin assay. The incorporation of an enzyme amplification method enhances the sensitivity of the assay thus permitting a shorter incubation time.

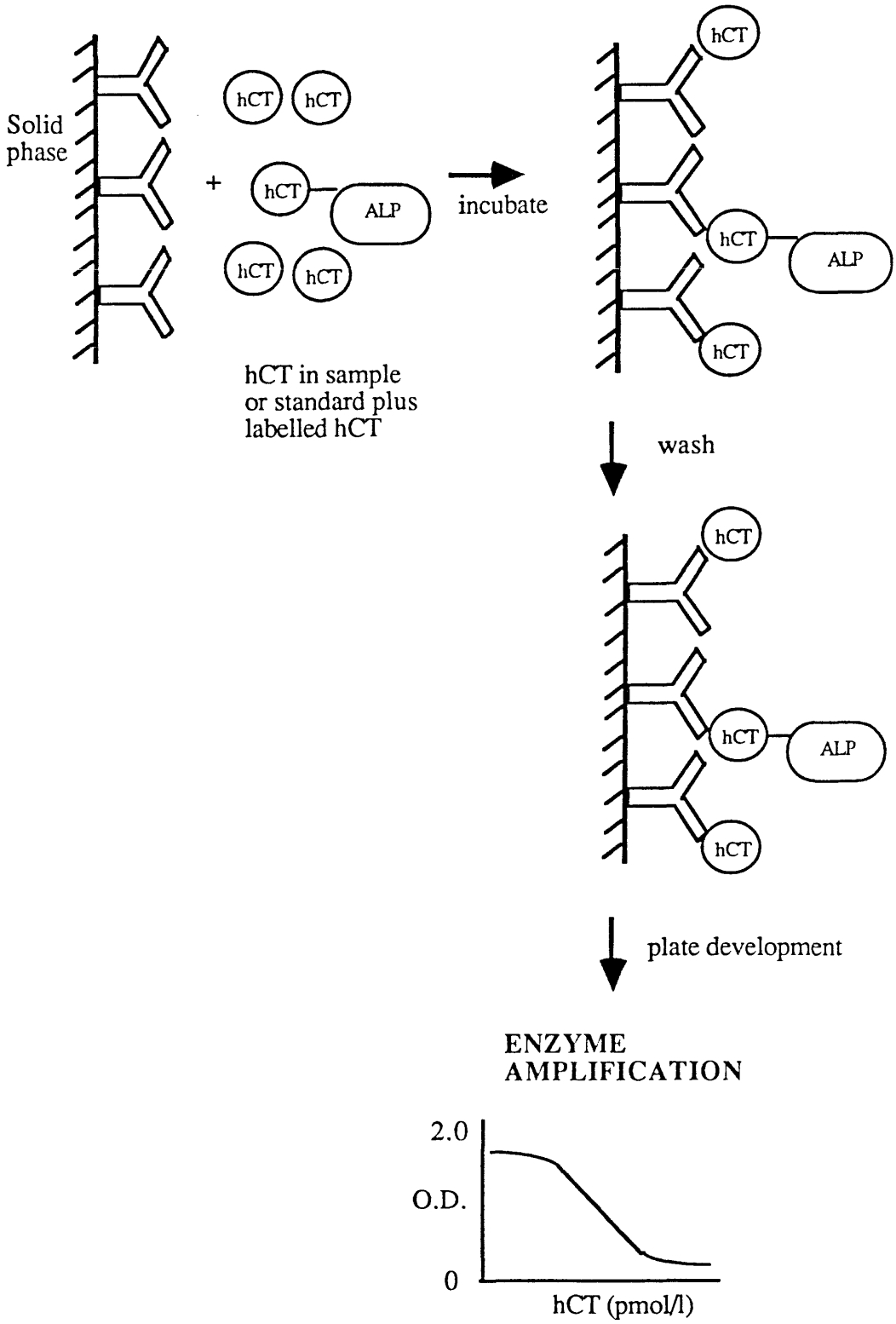


Figure 5: A one-site hCT assay format for hCT using polyclonal antibodies

2.3.2. Materials and methods for the one-site amplified enzyme-immunoassay (AEIA)

2.3.2.1. Components of AEIA - the solid-phase

A variety of solid-phases can be used in EIA. Its chemical nature may be glass (Engvall and Perlmann, 1971), nylon (Hendry and Herrmann, 1980), nitrocellulose paper (Hawkes, Niday and Gordon, 1982). However the most commonly used solid phase is polyvinyl or polystyrene (Catt and Tregear, 1967). The physical nature can be balls, rings, beads of varying capacity, and discs or sheets of paper. However, the majority of assays are performed with a solid-phase support in the form of a 96-well polystyrene or polyvinyl plate, which is especially suitable for large scale work.

2.3.2.2. Plate-coating

Antibody is passively adsorbed on to the solid surface as a result of hydrophobic interactions. The extent of coating depends on many factors such as temperature, volume of coating solution, and the nature of adsorbing substances. Early ELISA tests suggested that the antigen binds better at higher pH values. Therefore in this study, the pH of the coating solution (sodium carbonate, 0.02M/l) was 9.6.

2.3.2.3. Plate glazing

As most solid supports adsorb proteins non-specifically by hydrophobic interactions, it is evident that they will also adsorb the reagents non-specifically if the plates are not fully saturated. Therefore, once the antibody has been bound, the plates must be blocked. A number of reagents can be used for this purpose, including BSA, casein, gelatin, non-specific serum and bovine gamma globulin (all at about 1% w/v). The use of 0.01% v/v Tween-20 also reduces the background as it reduces the surface tension and discourages the formation of hydrophobic interactions between the solid-support and the reactants.

2.3.2.4. Storage of coated plates

Antibody coated plates can be stored at 4°C for several weeks. Sodium azide (0.02%) is usually included in the glazing buffer to avoid bacterial contamination.

2.3.2.5. Enzymes used in immunoassays

The sensitivity of an enzymeimmunoassay (EIA) is directly related to the signal imparted by the enzyme moiety. The following criteria must be met by an enzyme-analyte or antibody conjugate in order to achieve a sensitive, easily performed EIA:

a) the enzyme should be relatively stable at room temperature and have a shelf-life of at least six months at 4°C.

b) the purified enzyme should be available in a pure form and be relatively inexpensive.

c) the activity of the enzyme should be easily measurable using simple colorimetric methods.

d) the enzyme should have a high substrate turnover number, and therefore small amounts of it should be detectable.

e) the enzyme should not be affected by biological components of the test samples especially in the competitive EIA format.

Alkaline phosphatase was used in the present study since it satisfied all the above criteria. Its activity can be quantitated simply and sensitively using either p-nitrophenyl phosphate (pNPP) (spectrophotometric) or 4-methylumbelliferyl phosphate but the latter is detected at about 10^{-9}M compared to about 10^{-5}M for pNPP (Ishikawa, Imagawa, Hashida, Yoshitake, Hamaguchi and Ueno, 1983).

2.3.2.6. Enzyme Amplification

The detection method used in this study is known as enzyme amplification (Self, 1985). The principle of this approach is that the enzyme label gives rise to a catalytic activator for a specific secondary detection system, the activity of which is measured and related back to the amount of enzyme label present.

When alkaline phosphatase is used as the labelling enzyme and nicotinamide

adenine dinucleotide phosphate (NADP) is used as its substrate, the nicotinamide adenine diphosphate (NAD) formed is in turn reduced to NADH by ethanol catalysed alcohol dehydrogenase. NADH is then reoxidised by a tetrazolium salt catalysed by diaphorase and the reduced tetrazolium chromophore is then measured. Since NAD is regenerated, each molecule of NAD produced from hydrolysis of NADP is involved in many cycles of reduction of tetrazolium, thereby resulting in amplification of the reaction products of the enzyme labelled material (see figure 6).

2.3.2.7. Enzyme-labelled material

Human CT-alkaline phosphatase conjugate

The coupling of a peptide with a protein is usually performed with a bifunctional reagent and often results in a heterogeneous mixture of products. The basic problem in protein-protein coupling is controlling the stoichiometry, reagent selectivity, reaction conditions and other factors to obtain enzymatically and immunochemically active conjugates.

Many reagents are available for coupling and cross-linking (for review see Ishikawa *et al.*, 1983)

In the present study, the maleimide method was used to conjugate calcitonin and immunoglobulin G with alkaline phosphatase enzyme and used in the competitive EIA and in the two-site assay respectively. The conjugations were carried out by I.Q. Bio Ltd., Cambridge.

Basically, the maleimide groups react under mild conditions with thiol groups fairly rapidly and slowly with other functional groups which make them suitable cross-linking agents. N, N-o-phenylenediamine was used in this study. The analyte or antibody is first reduced with 2-mercaptoethanol to generate thiol groups and then treated with excess dimaleimide to introduce maleimide groups. The maleimide-protein complex is allowed to react with thiol groups introduced in the same way on the alkaline phosphatase.

The main advantage of this method is that in coupling of the antibody to the enzyme, the conjugation site can be predicted (SH-HS groups in the hinge

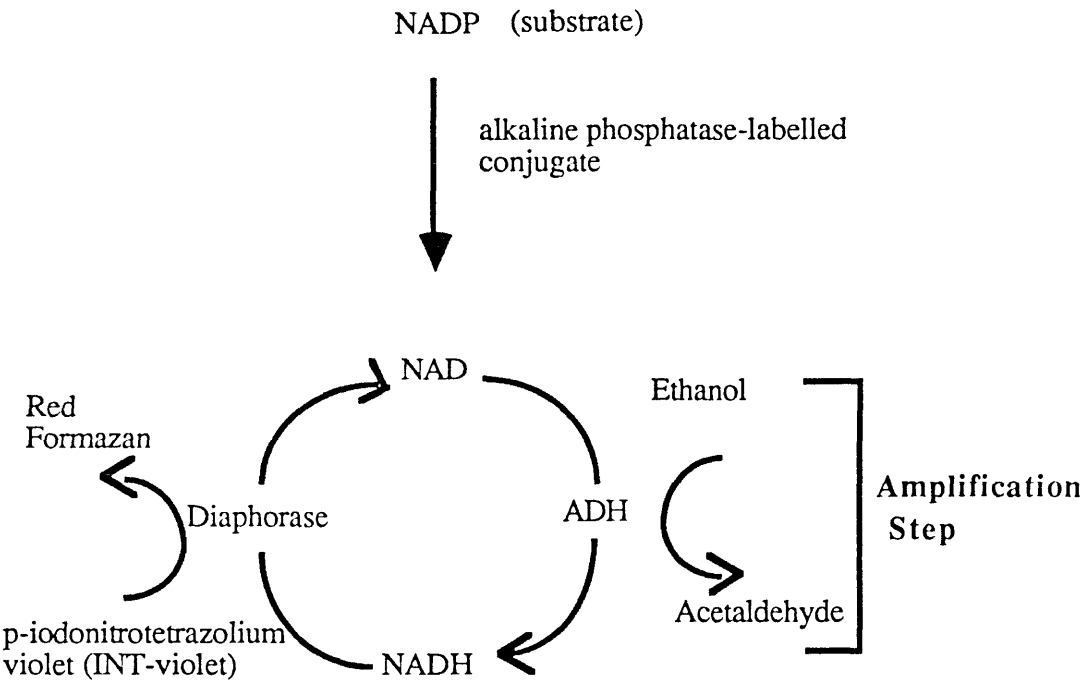


Figure 6: Diagrammatic representation of enzyme amplification triggered by alkaline phosphatase-labelled conjugate bound to the solid-phase antibody

region of the antibody) and is not involved with the antigen-binding sites. In the CT molecule, the coupling site is in the disulphide bridge. Another advantage is that it is highly reproducible and widely applicable to most kinds of peptides and proteins, with no self conjugation of enzyme, analyte or antibody.

A minor disadvantage is that the alkaline phosphatase loses 20-40% of its activity after the introduction of the thiol groups (Ishikawa *et al.*, 1983) and it is not applicable to conjugation with enzymes in which thiol groups are essential for the enzyme activity.

Purification of enzyme-labelled material is necessary to develop a good EIA. The presence of unconjugated analyte or antibody will lead to reduced sensitivity in all assays. In the present study, size exclusion HPLC gel-permeation was used to remove the labelling components.

The conjugate was prepared using the maleimide method as discussed before. Synthetic hCT(1μg/tube) was dissolved in 0.1M triethanolamine-HCl (pH 8.5) containing 0.1M sodium chloride and 1mM EDTA. Dithiothreitol (DTT) 10μl of a 50mM solution in water was then added and the reaction mixture incubated at 37°C for one hour. The reduced peptide was recovered by gel-filtration on a sephadex G-10 column in 0.1M triethanolamine-HCl (pH 7.3) containing 0.1M sodium chloride and 1mM EDTA. Calf-intestinal alkaline phosphatase (2mg) was reactivated by reaction with succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate for 15 minutes. The reduced peptide and the activated enzyme were coupled and recovered as described previously by Ishikawa *et al.* (1983) on size-exclusion HPLC.

The conjugate was tested for enzyme and immunological activity, aliquoted out, stored at -20°C and used at a final dilution of 1:25,000.

2.3.2.8. Antiserum

Anti-calcitonin antibodies (OC31)

Polyclonal antibody against hCT was raised as described earlier by Hillyard *et al.*, (1978) by repeated immunisation of three rabbits with an ovalbumin-conjugated hCT complex. A primary injection of 100µg per rabbit in Freund's complete adjuvant was administered into the hind footpads of each rabbit. Dorsal booster injections of 25µg of the conjugate were given in Freund's incomplete adjuvant subcutaneously six weeks after the primary injections and then at monthly intervals until a satisfactory titre of anti-calcitonin antibodies was obtained.

2.3.2.9. Radio-labelled peptide

The radiolabelling of the peptide was carried out by Mrs P. Hill using the Chloramine-T method and ^{125}I obtained from Amersham International (Greenwood, Hunter and Glover, 1963). The resulting label was purified using a reverse-phase gradient HPLC with a linear acetonitrile (ACN)/water/0.1% v/v TFA gradient, from 25% v/v to 45% v/v ACN. The resulting label was tested for binding using an excess antibody test.

2.3.2.10. Enzyme amplification kits

Enzyme amplification kits, containing washing, substrate, amplifier and stopping solutions (Ampaks) were purchased from Novo Bio Labs Ltd, Cambridge, U.K. and were used as instructed by the supplier.

2.3.2.11. Buffers

Coating buffer

Sodium carbonate (20mM) pH 9.6 was prepared as follows :

2.12g of sodium carbonate was dissolved in one litre of distilled water. The pH of the resulting solution was adjusted to 9.6 using 1M HCl.

Glazing buffer

To the above solution, the following compounds were added: casein (0.5% w/v), lactose (5% w/v), Tween-20 (0.02% v/v) and sodium azide (0.01% w/v).

Washing/conjugate-dilution buffer

Tris-HCl buffer pH 7.4, 50mM was prepared as follows:
7.9g of tris-HCl was added to one litre of distilled water. The pH was adjusted to 7.4 using 50mM Tris-base solution. 0.015 M sodium chloride and 0.1% w/v casein was added.

Phosphate buffer for RIA

Sodium phosphate buffer (0.05 M, pH 7.4) was prepared containing human serum albumin (0.025% w/v) and aprotinin proteinase inhibitor (Trasylol).

2.3.2.12. Human calcitonin standard

Synthetic hCT was a gift from Dr. W. Rittel (Ciba-Geigy Ltd, Basle, Switzerland). House standard of hCT was standardised against hCT (Ref 70/234) from NIBSC, Holly Hill, London. The house standard was then prepared as follows: to 800ng of peptide, 8ml of double-distilled water containing mannitol, acetic acid and BSA was added. The resulting solution was aliquoted out (100µl/tube), lyophilised and stored. Each tube contained 10ng/tube of the peptide.

The same standards were used for EIA and RIA as follows: serial dilutions of hCT were prepared in PPF ranging from 0-2000 pg/ml.

2.3.2.13. Plasma purified fraction

Plasma purified fraction (PPF) was obtained from Blood Products Laboratories, Dagger Lane, Elstree, Herts. It was used (as a zero standard) to prepare the calibration graphs for hCT assays developed for the measurement of plasma samples.

2.3.2.14. Assay development and optimisation of conditions

The following criteria must be satisfied in order to develop sensitive and robust assays.

a) optimal solid-phase antibody

b) conjugate dilution

c) calibration curve

d) sensitivity

e) specificity

f) intra- and inter-assay variation

g) recovery and parallel dilution

Each of the above is discussed below.

2.3.2.15. Optimisation of solid-phase antibody

This assay format is identical to the conventional radioimmunoassay. Thus sensitivity of an assay is increased by reducing the antibody concentration. Therefore, it was necessary to establish the optimum concentration of the coating antibody.

Anti-hCT antiserum (OC31) was serially diluted in the coating buffer in the following range and placed (100µl/well) in the micro-titre plate: 1/1000, 1/2000, 1/4000, 1/8000, 1/16,000, 1/32,000 and 1/64,000. The plate was incubated at room temperature overnight. At the end of the incubation time, the antiserum was shaken out and the plate washed four times with the glazing buffer (200 µl/well) to block the unbound sites on the plate.

Serially diluted hCT standards (80µl/well) in PPF were placed in the wells of the coated plate. Alkaline phosphatase-labelled hCT was then added to the plate (20µl/well) at an initial dilution of 1:5000 (chosen at random) in tris buffer and incubated at room temperature overnight.

At the end of the incubation, the reaction mixture was shaken out to remove the unbound labelled and unlabelled hCT and the plate washed four times with the washing buffer. The bound conjugated analyte was quantitated using the enzyme amplification method.

The reagents for enzyme amplification were purchased in a kit form and used as instructed by the supplier. Reconstituted substrate solution (100µl/well) was placed in all the wells and incubated for twenty minutes. Amplifier solution (200µl/well) was then added to all the wells and the colour produced was measured on a spectrophotometer (Titertek Multiscan). In such an assay the wells containing the zero standard have the most intense colour since there is no unlabelled hCT present to compete with the labelled hCT for the number of limited antibody sites on the solid-phase.

As soon as the O.D. of these wells reached 2 absorbance units, the enzyme reaction was terminated by the addition of 1M sulphuric acid (50µl/well) and the absorbance measured once again.

The optimum amount of antiserum coating was that which gave the most sensitive calibration graph and was found to be 1:40, 000 initial dilution at 22°C (see figure 7)

2.3.2.16. Enzyme-conjugate dilution

Using the constant amount of coating antiserum (1:40,000) the conjugate concentration was varied at 1:1,000, 1: 2,500, 1: 5,000 and 1:10,000 initial dilution.

The assay was carried out in the same manner as stated above and the plate was developed using the amplification system as before. The optimun dilution of conjugate which gave a sensitive calibration graph was found to be 1:5000.

2.3.2.17. The calibration curve and sensitivity

Serially diluted hCT standards ranging from 0-2000pg/ml (or 0-600 pmol/l, 80µl/well) in PPF were placed in the wells of the coated plate. Alkaline phosphatase-labelled hCT was then added to the plate (20µl/well) at an initial dilution of 1: 5000 (optimum dilution determined above) in Tris buffer and incubated at room temperature overnight.

At the end of the incubation, the reaction mixture was shaken out to remove the unbound enzyme-labelled and unlabelled hCT and the plate washed four

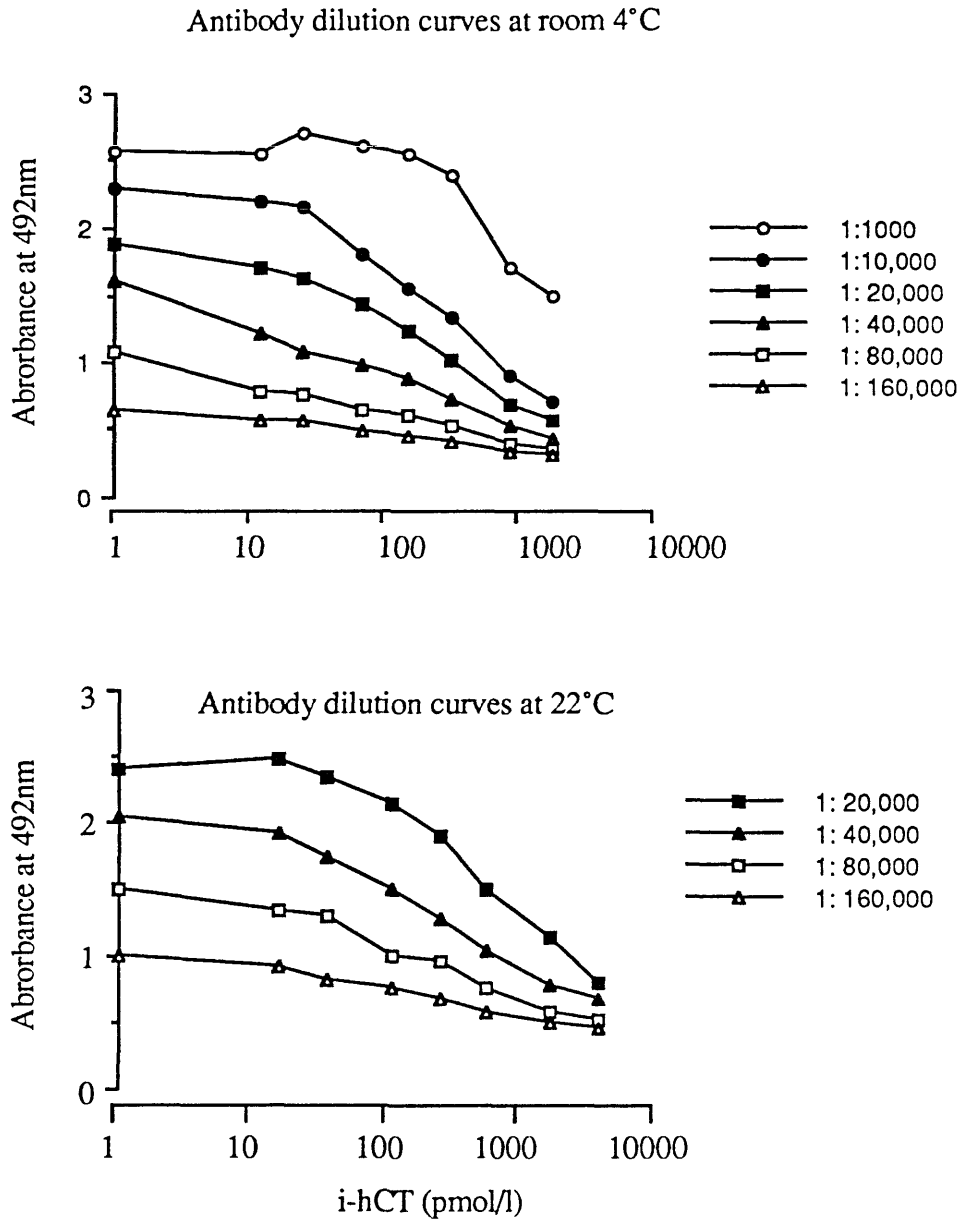


Figure 7: Effect of temperature on the solid-phase antibody (coating) at varying dilutions

times with the washing buffer. The bound enzyme-labelled hCT was quantitated using the enzyme amplification method. Figure 8 shows the standard curve obtained for comparison with the conventional RIA.

Sensitivity of an immunoassy is defined as the least amount of an analyte which can be detected in the test with 95% confidence (Wood and Sokolowski, 1981).

In the present study, the zero standard was measured twenty times in one assay. The optical density was averaged and the two and a half times the standard deviation value (2.5xSD) was determined. A calibration curve was plotted and the zero minus 2.5xSD value was measured on the graph. The sensitivity of the amplified enzyme immunoassay (AEIA) was determined as 6 pmol/l, whereas the sensitivity of the corresponding RIA was 20 pmol/l. Thus the enzyme immunoassay was found to be more sensitive than the RIA.

2.3.2.18. Precision and specificity

The performance of the assay was tested by preparing and assaying two quality control "pools" containing known amounts of hCT in PPF since the assay was developed for use in the measurement of hCT in human plasma. The low and high pools contained 73.1 and 292.6 pmol/l hCT. Each pool was assayed twenty times using different wells on single plates and then repeatedly in ten separate assays using different plates to obtain intra- and inter-plate coefficients of variation.

The specificity of an immunoassay depends on the ability of the antiserum to recognise and bind to the particular analyte of interest in the presence of other substances with similar structures and chemical properties. In order to check the specificity of an antibody, a calibration curve was set up with increasing concentrations of the possible cross-reacting substances in the concentration range of 10-100 fold higher than those expected under physiological conditions. The peptides that were tested for cross-reactions at dose levels of up to 100 fold greater than the synthetic hCT were:- salmon calcitonin, eel calcitonin, porcine calcitonin, katacalcin, rat CGRP, human CGRP, bombesin, follical stimulating hormone, luteinising hormone, thyroid stimulating hormone, growth hormone,

insulin, angiotensin ii, angiotensin iv, pre-opiomelanocortin (POMC), substance P, vasopressin and glucagon.

OC31 antiserum has previously been used in RIA and its characteristics published eleswhere (Hillyard *et al.*, 1978). It was found to be specific for hCT.

2.3.2.19. Recovery and Parallel dilutions

Plasma samples were obtained from ten patients with non-thyroid malignancies.Synthetic hCT was added to 1ml of plasma from each patient, and the samples were assayed before and after the addition of the synthetic hCT using AEIA. The mean percentage recovery of hCT was determined. The mean recovery of the added synthetic hCT (58.5 pmol/l) to the plasma of ten patients was found to be 110% (64.4 pmol/l).

Parallel dilutions of plasma from MTC patients were carried out to check the performance of the calibration curve. Figure 9 shows the parallel dilutions obtained with plasma from patients with high levels of CT.

2.3.2.20. RIA

The RIA used in the present study was a modification of the previously published method. Synthetic hCT house standard was standardised against human calcitonin (ref 70/234) from NIBSC, Holly Hill, London. Serial dilutions of hCT were prepared in PPF. Sample or standards (100µl) were dispensed with phosphate buffer (100µl). Antiserum OC31 (50 µl in phosphate buffer) was added to each tube to give a final dilution of 1:180,000. Radiolabelled hCT (50 µl containing 10,000 counts in 100 seconds) were added to all the tubes.

Following an overnight incubation at 4°C the antibody-bound radioligand was separated from the antibody-free form by immunoprecipitation with sheep anti-rabbit antibody bound to cellulose.

The radioactivity in the precipitate was quantitated on a gamma counter. A calibration graph obtained is shown in figure 8.

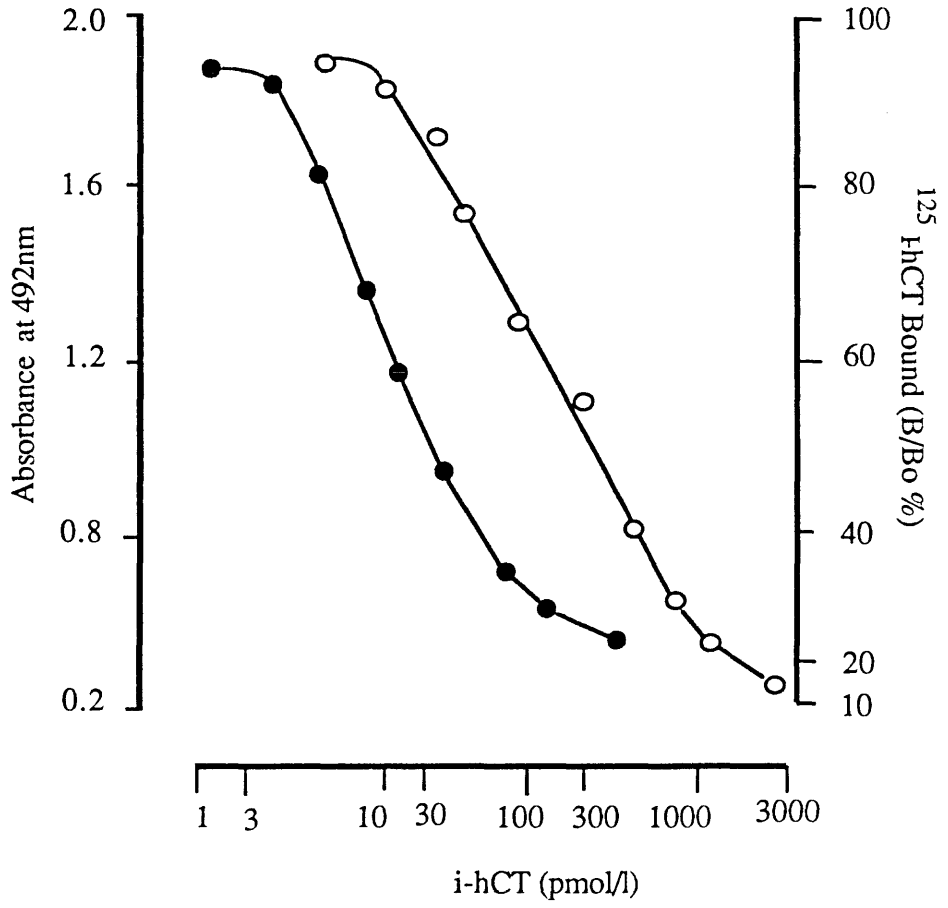


Figure 8: Calibration graph of hCT determination by one-site AEIA (●) and RIA (○) using the same polyclonal antibodies

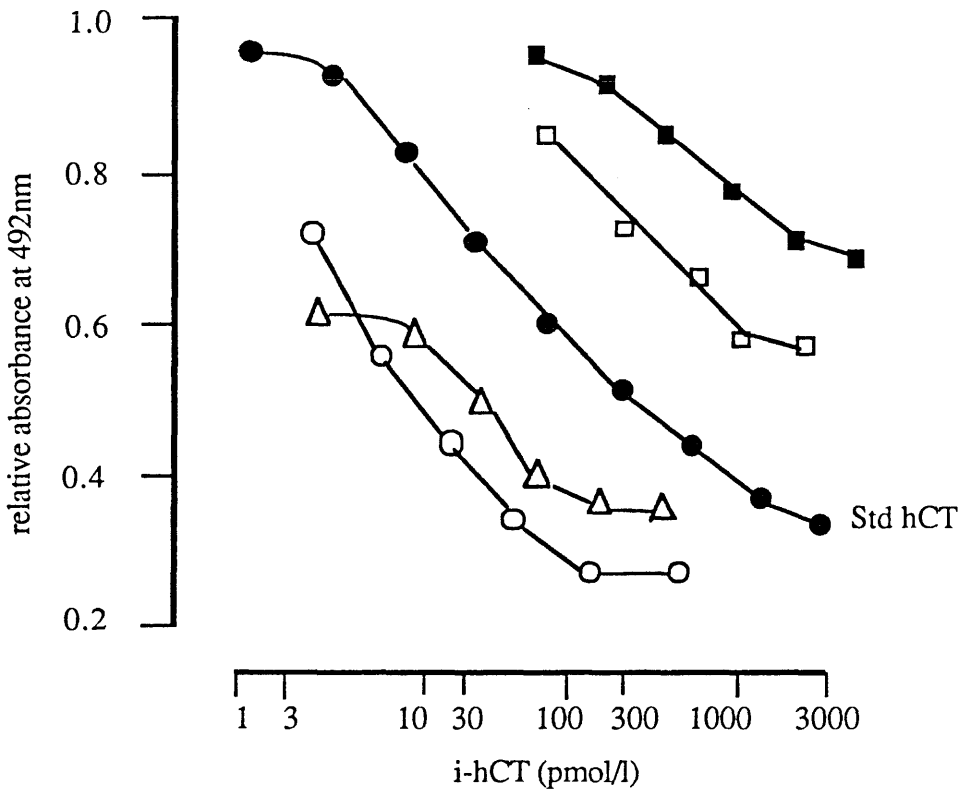


Figure 9: Comparison of dilution curves of immunoreactive hCT in plasma of MTC patients (○△□■) and synthetic hCT(●)

2.3.2.21. Correlation with clinical samples

Samples from various patients with medullary carcinoma of the thyroid (MTC) and non-thyroid malignancies were assayed by both RIA and AEIA. The results obtained were used to determine the correlation between RIA and AEIA.

The correlation coefficient between RIA and enzyme immunoassay was found to be 0.96 ($P < 0.001$). This meant that more than 80% of the samples gave the same readings in both the assays (see figure 10). The enzyme immunoassay was successfully applied to the measurement of calcitonin in patients with medullary carcinoma of the thyroid gland.

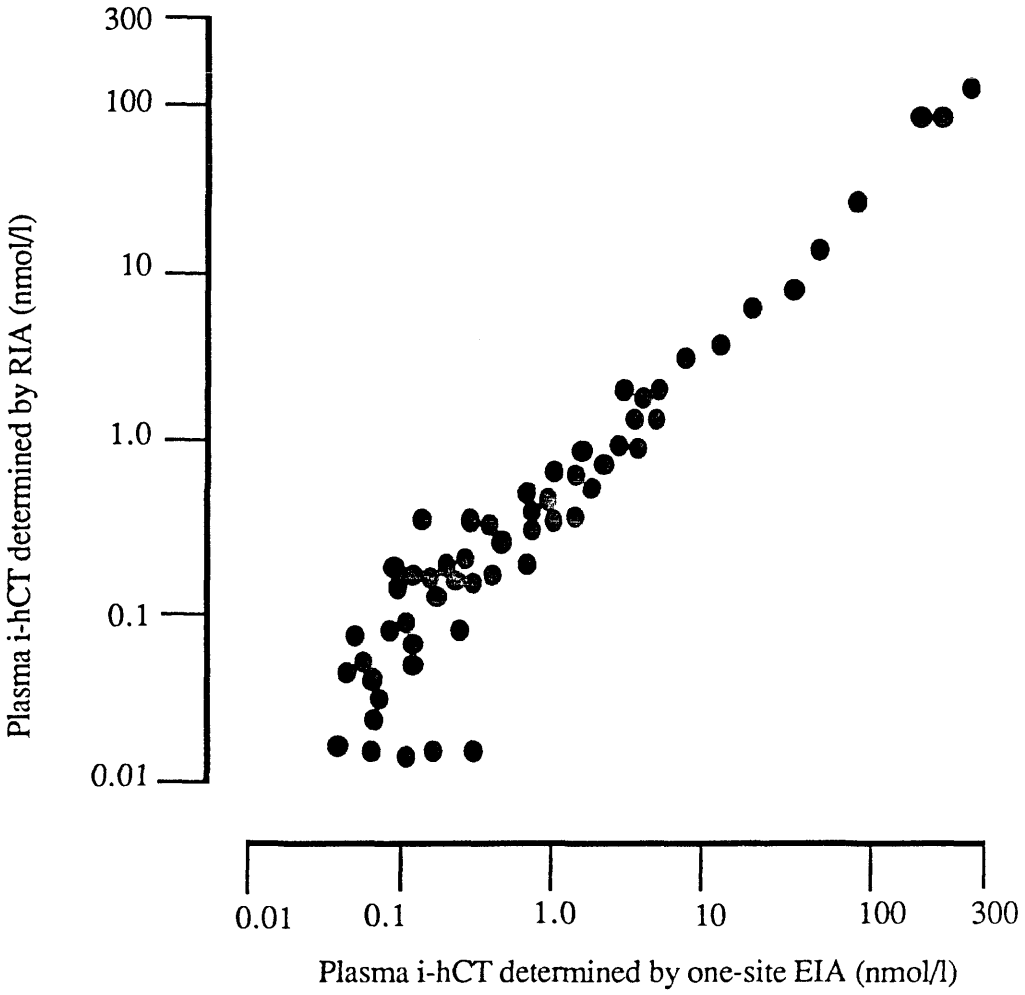


Figure 10: Correlation of plasma samples obtained from patients with MTC and non-thyroid malignancies determined by RIA and the one-site EIA

2.4. Non-competitive (two-site) immunoaassay for CGRP using polyclonal antibodies.

A non-isotopic immunoassay for rat CGRP has been reported previously using a competitive ELISA format (Self, Wimalawansa, Johannsson, Bates, Girgis and MacIntyre, 1985). Rat CGRP was labelled with alkaline phosphatase and an enzyme amplification method was used to enhance the sensitivity of the resulting immunoassay. It has been shown that a two-site approach provides higher sensitivity than conventional immunoassays (Ekins, 1985). This has been confirmed with a variety of assay formats and labels.

To date, no immunometric (two-site) assay for CGRP has been developed. In the present study of the development of more sensitive assays, the two-site approach was attempted. Polyclonal antisera, which had been raised for the studies on the physiologial importance of circulating CGRP, were used to construct a two-site assay (see figure 11).

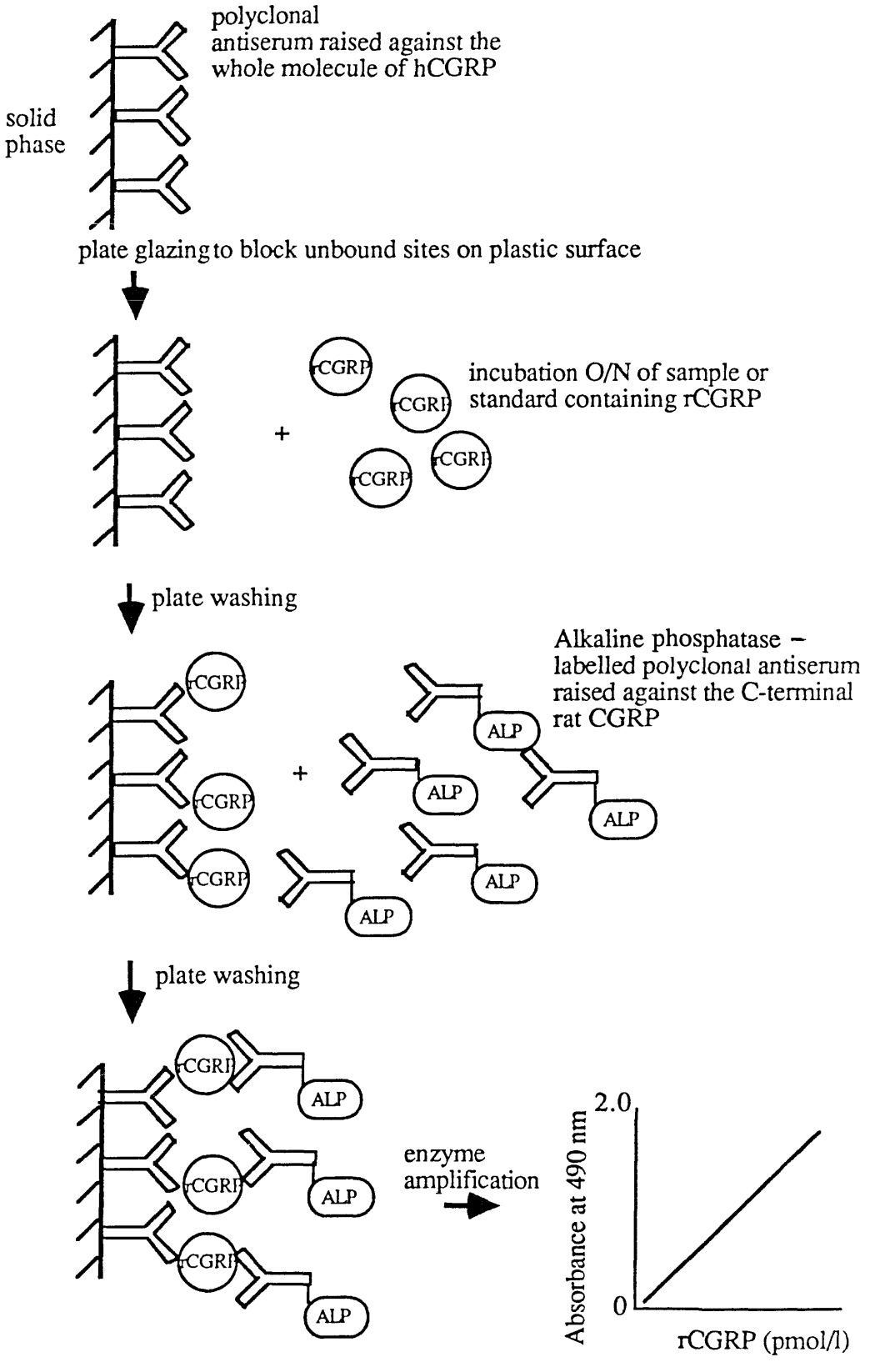


Figure 11: Two-site assay format for CGRP using polyclonal antibodies

2.4.1. Materials, methods and results for a two-site assay using polyclonal antisera

2.4.1.1.Antisera

Anti-human α-CGRP antiserum (CC21)

The polyclonal antibody against hCGRP was raised in a similar manner to the anti-calcitonin antibody, the characteristics of which have been reported elsewhere (Girgis *et al* .,1985). It was referred to as 'whole molecule' antibody since it did not cross-react with several small CGRP fragments.

Anti-Rat C-terminal CGRP antiserum (CG39)

Rat C-terminal antibody was raised in the same manner in rabbits against the 28-37 tyrosinated decapeptide conjugated with BSA by the glutaraldehyde method (Sachs and Winn, 1970). No cross-reactions were found with calcitonin, katacalcin, enkephalins, PTH, oxytocin, GRF, cholecystokinin, synacthen, bombesin or N-POMC. Human CGRP was found to cross-react 100% with the antibody and its application has been reported elsewhere (Zaidi *et al.*, 1985).

2.4.1.2 CGRP standard

Rat and human CGRP standards were prepared in a similar manner to CT standards and used in RIA and the two-site AEIA.

2.4.1.3. Solid-phase antibody

The optimum coating experiments for the plates for a two-site assay are not necessary since the solid-phase antibody is always in excess. However, it is necessary to economise on the antibody and so an experiment was performed to determine the optimum antibody dilution.

The solid-phase antibody used was CC2/1, raised against the synthetic hCGRP. An optimum antibody coating experiment was carried out in a manner

similar to that for the one-site CT assay and it was found that 1:2000 final dilution was suitable for use with an overnight incubation at 22°C.

2.4.1.4. Enzyme labelled antibody

The rat C-terminal antibody (CG39) was used as the labelled antibody. This was purified on a protein A sepharose column to obtain the immunoglobulin of the G class prior to conjugation with alkaline phosphatase.

Conjugate fractions obtained during HPLC purification were tested. After the coating procedure, the plates were glazed as before. A two-fold duplicate dilution of rat CGRP standard was made in Tris buffer (0.050M, pH 7.4) containing sodium chloride (0.015M) and bovine serum albumin (0.1% w/v). Each fraction (numbers 3-6) of the alkaline phosphatase conjugated antibody CG39 diluted in assay buffer (1/100, initial dilution,100µl/well) was placed in the microtitre plate and incubated overnight. The plate was then washed and developed using enzyme-amplification as stated previously.

From the results, it was clear that the fraction which gave the best result was fraction 3 as it probably contained the highest enzyme-antibody ratio (see figure 12).

2.4.1.5. Effect of different glazing buffers

The effect of two different glazing materials (casein and gelatin) were then investigated using the same procedure as above and using fraction 3 as the conjugate.

Gelatin was not a good glazing agent as it gave a higher blank value in the absence of the solid phase antibody (see figure 13). This was due to the non-specific binding of rCGRP to the glazing agents themselves and not to the solid-phase antibody. This figure also shows the high dose hook effect which is frequently observed in the two-site immunometric assay. Casein was used throughout this work.

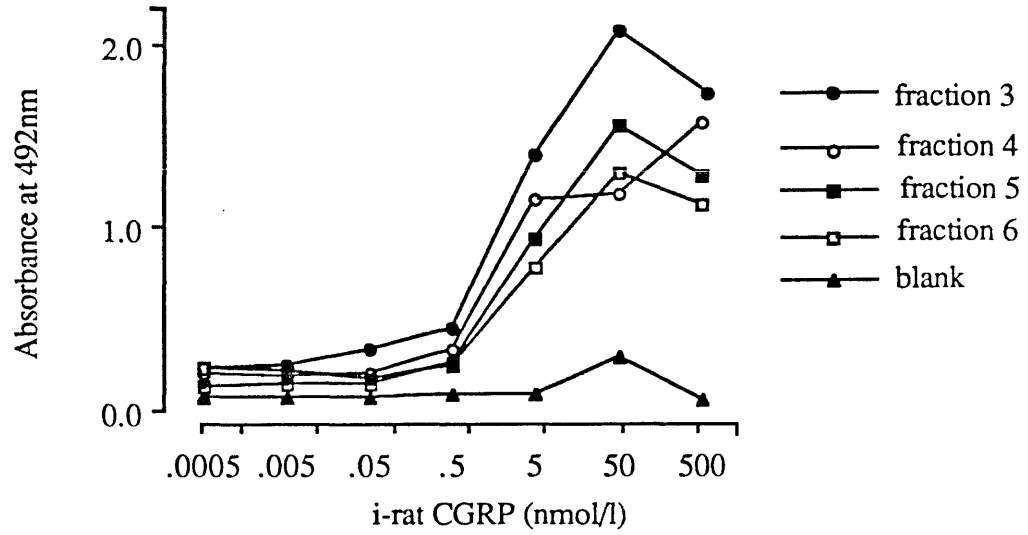


Figure 12: Optimisation of the antibody-enzyme conjugate following its purification after the coupling reaction. The effect of using high concentration of rCGRP in a calibration graph gives a 'high-dose hook effect'.

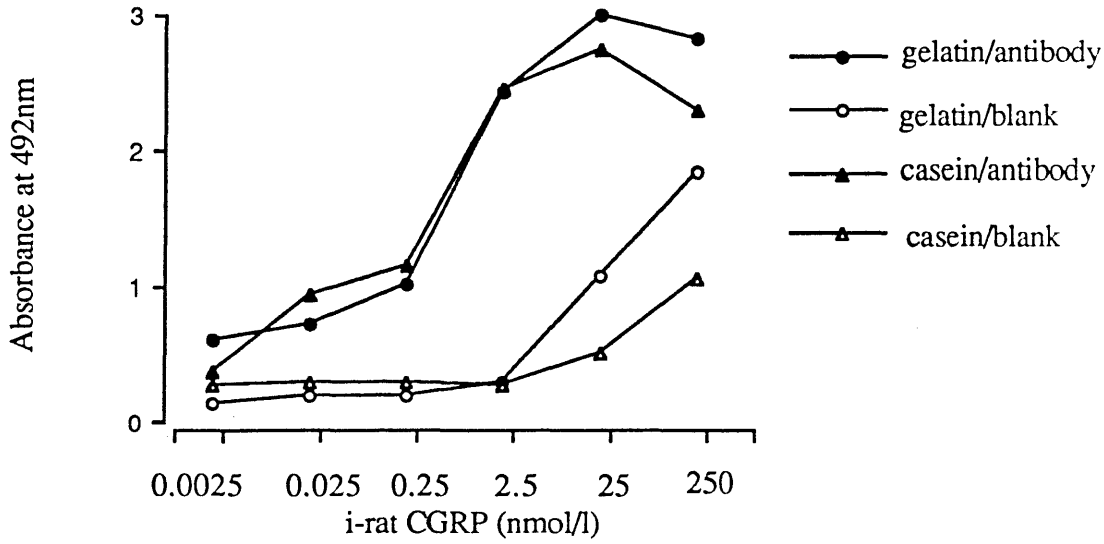


Figure 13: The effect of different glazing buffers on the rat CGRP (two-site) assay.

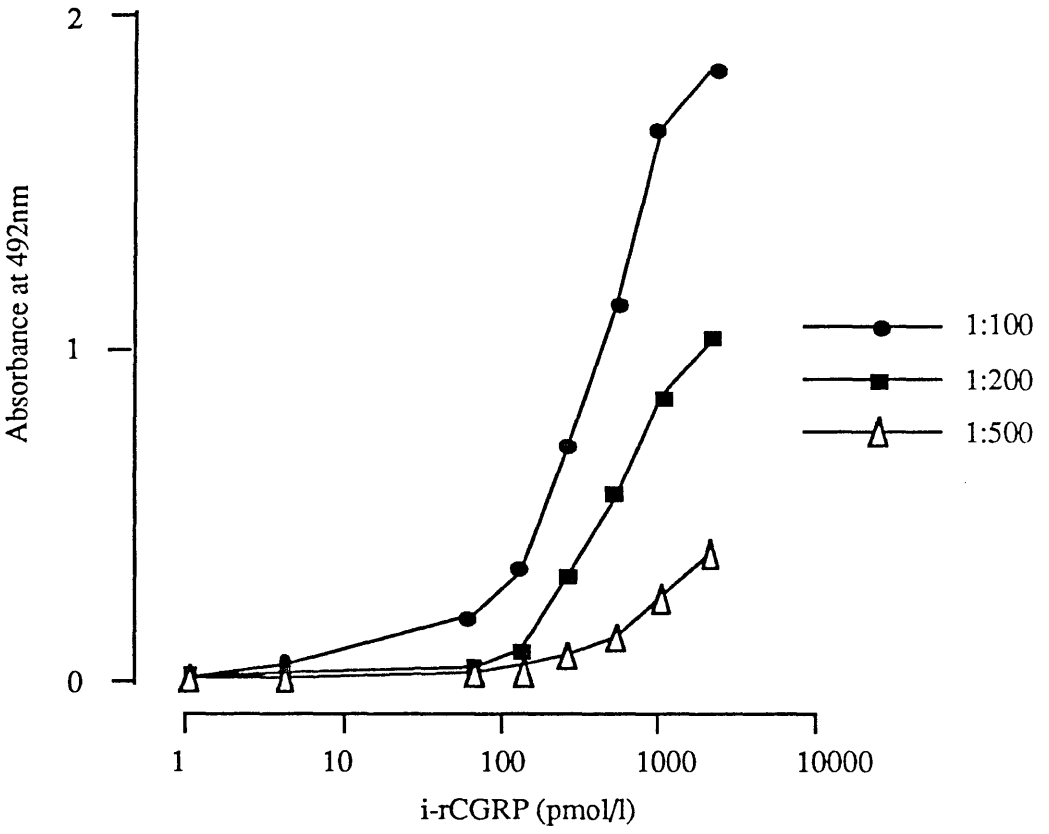


Figure 14: The effect of varying the dilution of the labelled antibody on the assay of rat CGRP

2.4.1.6. Calibration graph

Two-fold duplicate dilutions of the synthetic rat CGRP standard were prepared in assay buffer.Two quality control pools containing 6.25 and 12.5 pmol/l rat CGRP were prepared and used in all the assays. Duplicate dilutions of the standards and pools were dispensed into the wells of the coated plate and incubated for either two hours or overnight at room temperature.

Following incubation, the reactants were removed by shaking and the plate was washed four times with washing buffer. 100µl of alkaline phosphatase-labelled CG/39 (initial dilution 1:100) was placed into all the wells and incubated for 2 hours at room temperature. At the end of the incubation time the plate was washed four times with the washing buffer and developed as before.

Figure 15 shows the comparison of the conventional RIA with the overnight EIA and the parallel dilution effect of samples indicating that the assay is specific for rCGRP.

The assay sensitivity was found to be 0.25 fmol/tube on the overnight incubation. Table 5 shows the comparative sensitivity obtained on the RIA. Samples containing high amounts of CGRP were found to give parallel dilutions (see figure 15).

The assay was validated as before in terms of sensitivity, specificity, precision and recovery.

Sensitivity, specificity and precision

Assay sensitivity, as defined earlier, was determined at various incubation protocols by measuring the standard zero twenty times in the particular incubation protocol, averaging the absorbance and adding 2.5 times the standard deviation value to the mean followed by interpolation on the X-axis. The assay sensitivity was found to be 0.25 fmol/tube on the overnight incubation protocol.

Sensitivity (pmol/l)	AEIA	RIA
2hours	25	---
Overnight	2.5	40
2+1 days	---	5
3+2 days	---	2.5
Intra-assay variation (%)	6	10
Inter-assay variation (%)	10	10

Table 5: Summary of the characteristics of the polyclonal antibody-based RIA and the two-site AEIA for CGRP

Specificity	AEIA	RIA
rCGRP	100	100
hCGRP (α)	70	77
hCGRP (β)	70	166
1-11hCGRP	<0.1	<0.1
1-16 hCGRP	<0.1	<0.1
1-18 hCGRP	<0.1	<0.1
17-37 hCGRP	60	40
19-37 hCGRP	<0.1	11

Table 6: Cross-reactivity of the peptides and the sub-peptides used in the two-site AEIA for CGRP

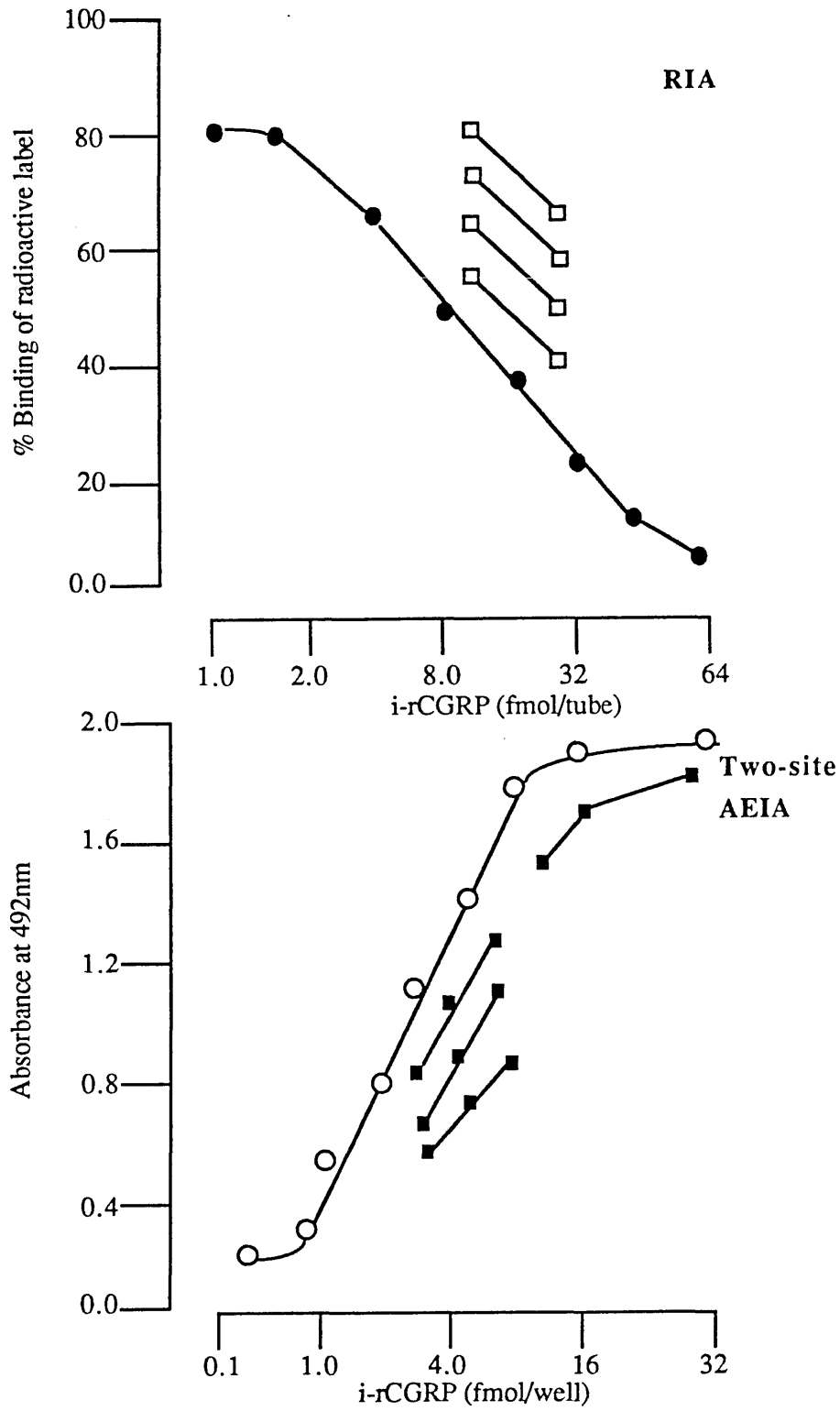


Figure 15: Comparison of the rCGRP calibration graphs obtained by RIA (●) and the two-site AEIA ○) showing parallel dilutions of immunoreactive rCGRP in plasma extracts. The open squares (□) indicate parallel dilution of samples by RIA whereas closed squares (■) indicate parallel dilution of samples by EIA.

A number of hCGRP analogues as well as unrelated peptides, were tested for cross-reactivity by both the RIA and the two-site AEIA at different molar concentrations. The results obtained are shown in table 6. Other peptides which were tested and did not cross-react at dose levels of up to 1nmol/l were bombesin, ACTH, sCT, hCT, katacalcin, glucagon and substance P.

From the assay of quality control pools prepared as before, the within-assay and between assay variations were obtained. They were 6% and 10% respectively.

To determine the precision of the assay, dose-error relationships were constructed from duplicate dilutions of standards and samples as shown in figure 16. The working range of the assay was found to be between 2 and 20 fmol/tube.

2.4.1.7. RIA

An RIA using antiserum CG39 has already been described by (Zaidi *et al.*, 1985)

2.4.1.8. Samples

The assay was applied to the measurement of CGRP in normal rats, and rats treated with capsaicin, a powerful vasoactive neurotoxin, to investigate further the neural release of CGRP. Forty-day male Wistar rats (n=15, mean weight 150g) were used. The animals were fed normally and only starved 18 hours before the experimental procedures. The animals were exsanguinated under light ether anaesthesia, via the dorsal aorta. Plasma was separated and frozen within 30 minutes of collection. A second group of animals were treated with capsaicin dissolved in Tween 80: ethanol: normal saline (1:1:8) injected intraperitoneally (10mg/kg). The rats in this group were kept sedated throughout the procedure with pentobarbitone (0.2 mg/kg, intraperitoneally).The group was matched with a vehicle-treated group. The rats were exsanguinated 7.5 minutes following injection.

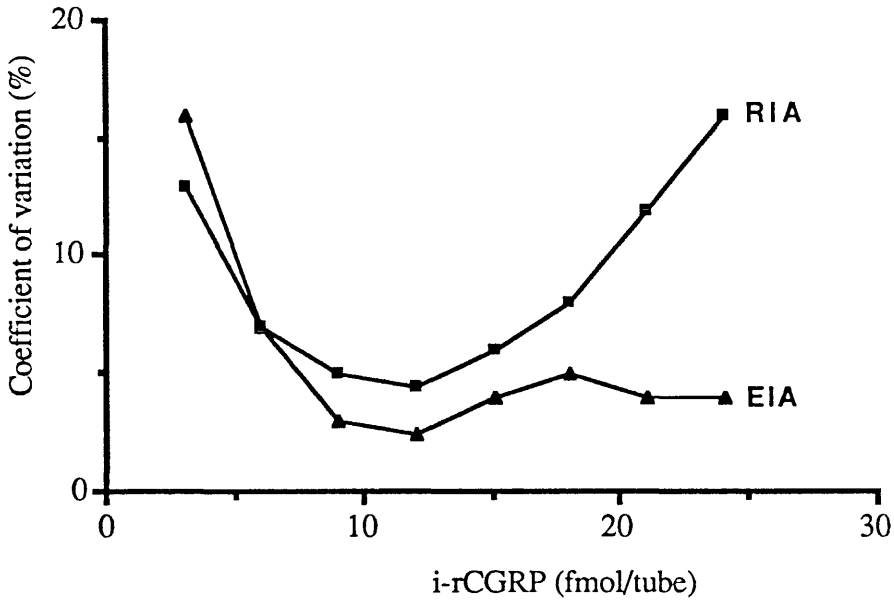


Figure 16: **Dose-error relationship for immunoreactive rCGRP constructed by plotting the coefficient of variation (%) of replicate measurements of standards from ten dose-response curves against each concentration to give a working range between 2 and 20 fmol/tube**

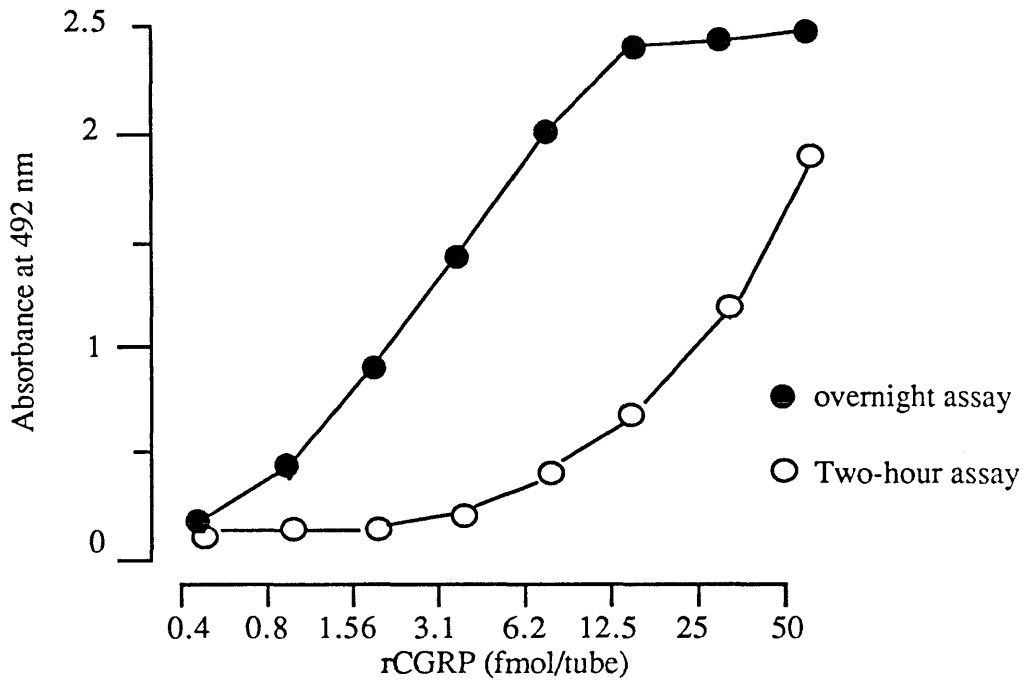


Figure 17: The effect of reducing the incubation time on the sensitivity of the two-site assay AEIA

2.4.1.9. Extraction of CGRP

The extraction process, modified from Bennett's protocol (Bennett, Hudson, McMartin and Purdon, 1977) was essentially as follows. Plasma was acidified with a medium comprising formic acid (5% v/v), trifluoroacetic acid (TFA, 15% v/v), hydrochloric acid (1M), mercaptoethanol (0.5 M) and sodium chloride (1% w/v). Following one-hour incubation of the sample with the above medium, centrifugation was carried out for 15 minutes at 500g. The supernatants were passed three times through prewetted sep-pak C18 cartridges (Waters Associates, Milford MA, USA.).

The peptide was finally eluted in aqueous methanol (80% v/v) containing TFA (0.5% v/v). The eluate was vacuume-dried and reconstituted in phosphate buffer on the day of assay. The mean recovery of synthetic CGRP (Peninsula laboratories UK Ltd) added to charcoal stripped plasma was 75 ± 3.6 % (by RIA) and the variation between the extractions was < 15%.

The samples were assayed for CGRP as follows. Duplicate dilutions of samples (50µl and 100µl) and standards were dispensed into coated and glazed plates and incubated either for two hours or overnight. Following incubation, the plates were shaken to remove the samples and the standards. The plates were washed as before and the enzyme-linked antibody (100µl/well) was added to all the wells. Following a further incubation for two hours the plates were washed and developed using the amplifer, as previously described.

2.5.1.10. Application of the assay

The assay was successfully applied to the measurement of CGRP in normal rats and rats treated with capsaicin.There was only a weak correlation ($r=0.57$; $p<0.01$) between values obtained by the two assay methods (see figure 18). This might reflect variability in the pattern of various molecular forms of CGRP in individual samples.

Figure 19 shows the results obtained using both methods. The mean CGRP levels of both normal and capsaicin treated plasma was 3 times lower when measured by EIA than the RIA.

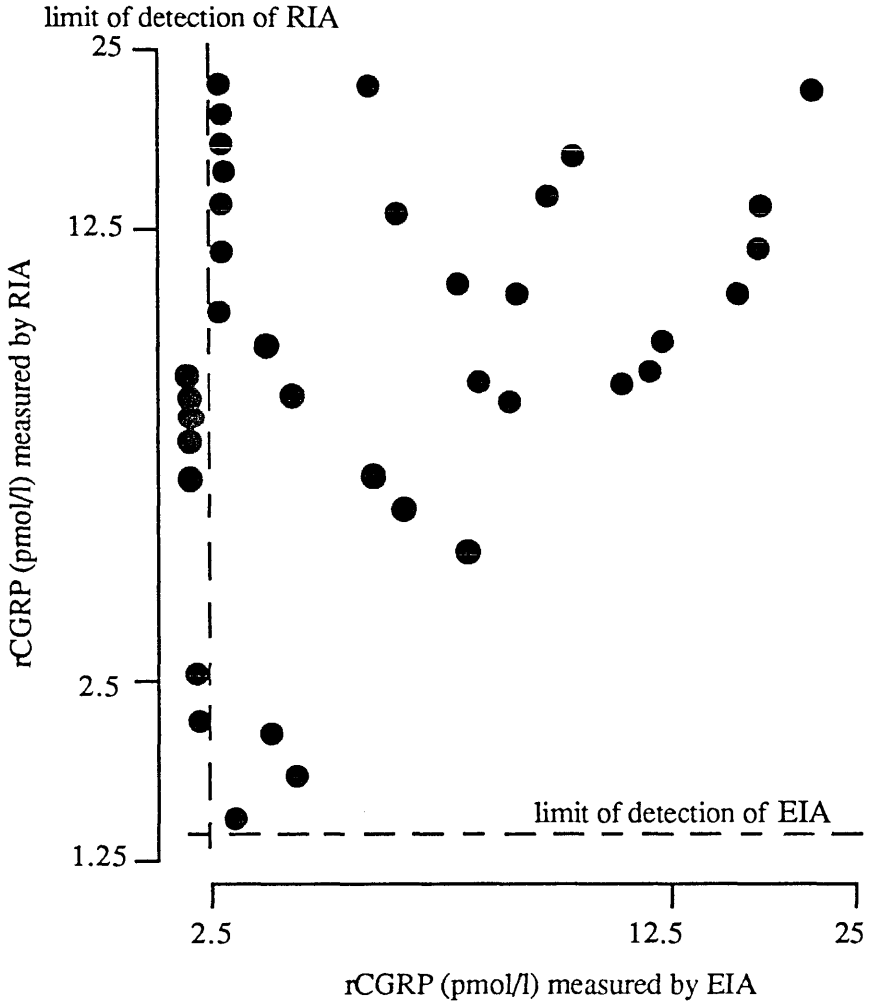


Figure 18: Correlation (r = 0.57, p< 0.1) between rCGRP levels as measured by RIA and the two-site AEIA

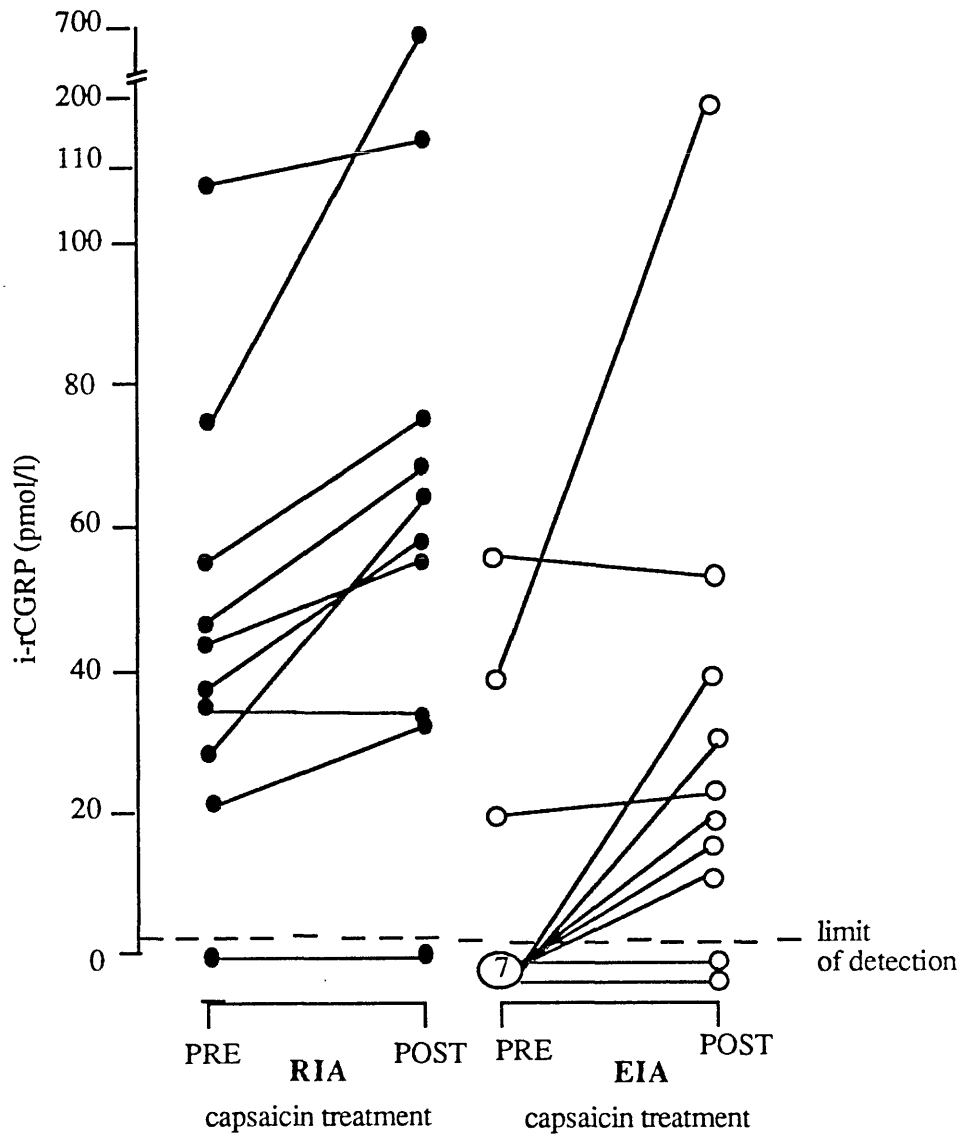


Figure 19: Measurement of immunoreactive rCGRP by RIA and AEIA before and after capsaicin treatment

2.6. Discussion

The earliest attempt to develop a non-isotopic assay for calcitonin was by Robertson in 1986 in the form of a competitive inhibition assay. This format was not suitable for the current study since there were many steps involved. This would not only have increased the time taken to perform the assay, but also introduced more technical errors. The single-site assay for calcitonin described in the present study had only one incubation step in which the samples or standards were added together with the labelled peptide on to the antibody coated plates. There was, therefore, direct competition for the antibody binding sites between the enzyme-labelled peptide and the peptide in the sample/standard. This provided a direct comparison with the conventional RIA in use at present which employs the same antibody.

The quantification of the bound analyte was made simply by directly adding the enzyme substrate after washing the plate. Incorporation of the enzyme amplification method enhanced the signal produced by the enyme-substrate reaction thus improving the sensitivity of the assay five times compared to the RIA. The assay was specific for hCT since plasma samples containing elevated levels show parallel dilutions.

The performance of all competitive EIA's depend greatly on a good separation of specific signal from noise. It was therefore particularly necessary to achieve good antibody coating at a constant temperature to avoid drift in the assay particularly in the wells situated on the edges of the plate. The dilution of the antibody required to achieve a sensitive assay was very high (1:40,000) since the antibody titre was high. It was found that by pre-coating the plates with donkey anti-rabbit antibody, followed by normal binding with anti-calcitonin rabbit antibody the resulting coating was much more uniform (intra-assay variation less than 5%; unpublished personal observations). However, this strategy was not employed since the inter - and intra-assay variations obtained without this method were acceptable.

The microtitre plate format used also added to the great convenience in that

the samples may be loaded on to the plates using multichannel pipettes thereby avoiding drifts in the assay which often occur in RIA's, and the developed plates read quickly using plate readers.

The speed of this assay could be useful in a clinical sense. In patients with MTC where CT levels are highly elevated it is possible to reduce the assay incubation time to just a few hours and obtain the results within a day. In the assay of plasma from patients with non-thyroidal malignancies or in normal volunteers the fact that the assay is capable of determining CT levels within this range without long incubation periods or extraction procedures was important.

The major drawback of this assay, as with any other single-site assay using polyclonal antibodies, is that it is not clear which epitopes are recognised by the antibody. Also, since plasma calcitonin exists in a heterogeneous form it was not clear which forms were being recognised in this particular polyclonal assay. For this reason it was necessary to develop monoclonal antibody-based assays where the epitope recognition sites are well characterised.

The enzyme amplified two-site assay for rat CGRP that was developed in the present study was found to be sensitive and specific. However, it must be emphasised that the extraction of the peptide was necessary prior to its quantification. The overnight incubation sensitivity was 2.5 pmol/l. When such high sensitivity is not required, it is possible to perform the assay with a 2-hour incubation period and still obtain the sensitivity and precison given by previous RIA. Such an assay is of particular use in monitoring CGRP levels during, for example, purification procedures.

The assay can be adapted to measure human CGRP (providing an extraction procedure is carried out beforehand) and this assay has a sensitivity of 20pmol/l. The two-site assay appeared to be more specific than RIA in terms of cross-reactivity with fragments of CGRP since two antibodies were used to detect the presence of the peptide. As there was also a wash step after incubation of the samples and prior to the addition of the labelled antibody, this enabled the removal of interfering substances.The cross-reactivity of 60% obtained with fragment 17-37 hCGRP reflects the contamination of that particular fragment

with the whole molecule during its synthesis.

The assay has been applied to the measurement of rCGRP before and after treatment with capsaicin, a potent neurotoxin. The mean plasma levels obtained by EIA were somewhat lower than those measured by RIA and there was only a weak correlation between values obtained, which may reflect variability in the pattern of the molecular forms in individual samples. The overall high performance of this assay was somewhat surprising since it had been constructed with polyclonal antisera which were at hand, and so the development of this two-site assay was an exercise to see if an immunometric assay could be developed for a small peptide such as CGRP.

It is likely that vast improvements can be made by affinity purification of the antibody as carried out by Hodgkinson, Allido, Landon and Lowry (1984), or by producing monoclonal antibodies directed at specific epitopes and eventually developing a more specific two-site assay for this small peptide.

SECTION III

Chapter 3: Monoclonal antibody production and characterisation

Summary

Monoclonal antibody production was initiated for hCGRP and hCT in order to examine the extent to which the theoretical advantages of monoclonal antibodies may be realised in practice.

The peptides were conjugated to ovalbumin using glutaraldehyde and the resulting complexes were used to immunise mice. Cell fusions were carried out using polyethylene glycol as the fusogen, and after HAT selection and screening procedures, the positive clones were expanded in vitro *and then* in vivo. *Ascitic fluids were tested for antibody titre and compared with that of the existing corresponding polyclonal antisera sera raised in rabbits. Isotype determination revealed mainly the IgG_1 class of antibodies for hCT whereas IgG_1, 2a and IgM were identified for CGRP. A number of synthetic sub-peptides of hCT were used for epitope mapping and it was found that the C-terminal proline amide residue was necessary for antibody binding. As predicted from epitope determination charts, the C-terminal region of the hCT molecule was highly immunogenic.*

Although five possible epitopes were located for alpha- hCGRP, the immunogenic response of this peptide was very weak. One possible explanation may be that the tertiary folding of the alpha-CGRP molecule is such that it renders it less immunogenic than calcitonin.

Chapter 3: Monoclonal antibody production and characterisation

3.1. Introduction

The monoclonal antibody production technique was first described in 1975 by Kohler and Milstein when they fused β-lymphocytes (antibody producing cells) with a myeloma cell line, which possessed the property of immortal growth in culture. The resulting hybridomas allowed immunoglobulin producing cells to be immortalised.

Monoclonal antibodies offer several advantages over conventional polyclonal antisera in the context of peptide hormone immunoassay. The availability of large quantities of defined antibody with constant characteristics allows better standardisation of assays. The greatest advantage of monoclonal antibodies is their use for the development of two-site immunometric assays because specific monoclonal antibodies can easily be purified and can then readily be conjugated to an enzyme or an isotope, leading to the development of immunoassays with great specificity (Comitti, Racchetti, Gnocchi, Morandi and Galante, 1987).

In order to examine the extent to which this advantage of monoclonal antibodies may be realised in practice, a programme of work was undertaken for human calcitonin (hCT) and calcitonin gene-related peptide (hCGRP). A method of antigenic epitope determination (Hopp and Wood, 1981) was used to predict the number of possible antigenic regions on hCT and hCGRP molecules. This method is based on plotting the hydrophilicity factor of each amino-acid against its sequence in the peptide and locating the turns in the peptide as shown in figures 20 and 21. Three possible sites for hCT and five for hCGRP were located. Monoclonal antibody production (Galfre and Milstein, 1981) was then initiated to produce antibodies which react with two different and distinct epitopes on these small peptides.

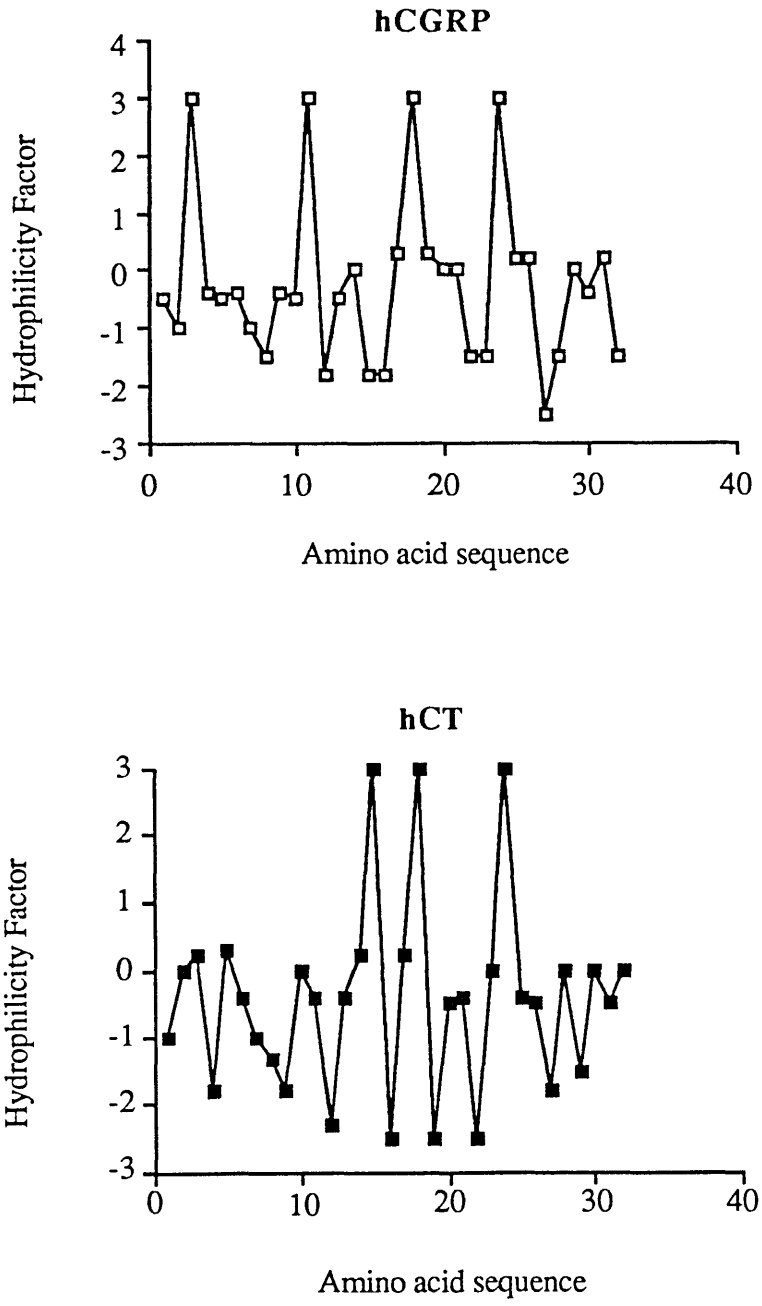


Figure 20: **Hydrophilicity profile of hCGRP and hCT shows the similarity in the peptide structure which allows them to interact at the receptor level**

3.2. Materials and methods

3.2.1. Immunisation protocols

3.2.1.1.Preparation of immunogens

The methods of preparing the conjugates and their subsequent immunisation protocols are numerous, so no method can be given as 'the rule'. In this study, hCT and hCGRP were coupled to ovalbumin using glutaraldehyde as the coupling agent (Sachs and Winn, 1970).

The peptides (250 μg) were dissolved separately in phosphate buffered saline (0.5ml). Ovalbumin was dissolved in the same buffer (4mls) and mixed with the peptides. Radiolabelled hCT and hCGRP (50,000 counts per minute) was also added to the reaction vessel to monitor the reaction. Glutaraldehyde (10% v/v, 0.4ml) was added dropwise and the reaction vessel was left on ice for one hour. A further 200μl of glutaraldehyde was added and the reaction was terminated by adding 400μl of saturated sodium sulphite solution.

The reaction mixture was placed on a sephadex G-25 column, developed in 0.2M ammonium acetate buffer (pH 6.4), to purify the peptide-protein conjugate. Fractions (5ml) were collected and the radioactivity present in all the fractions was quantitated in a γ-counter. The conjugated peptide eluted in the void volume (see figure 21). The fractions which contained the highest radioactivity in the void volume region were pooled and freeze-dried.

3.2.1.2. Immunisation of animals

Female mice (CBA x Balb/C, F1 hybrids, 6 weeks old) were used since the myeloma cell line was from Balb/C. 100μg of immunogen containing approximately 10μg of hCT were dissolved in 1ml of saline. An equal volume of complete Freund's adjuvant containing heat killed mycobacteria was taken and the solution containing the immunogen was added with rapid mixing to produce a stable water-in-oil emulsion.

The technique of emulsification employed two 5ml syringes joined by a

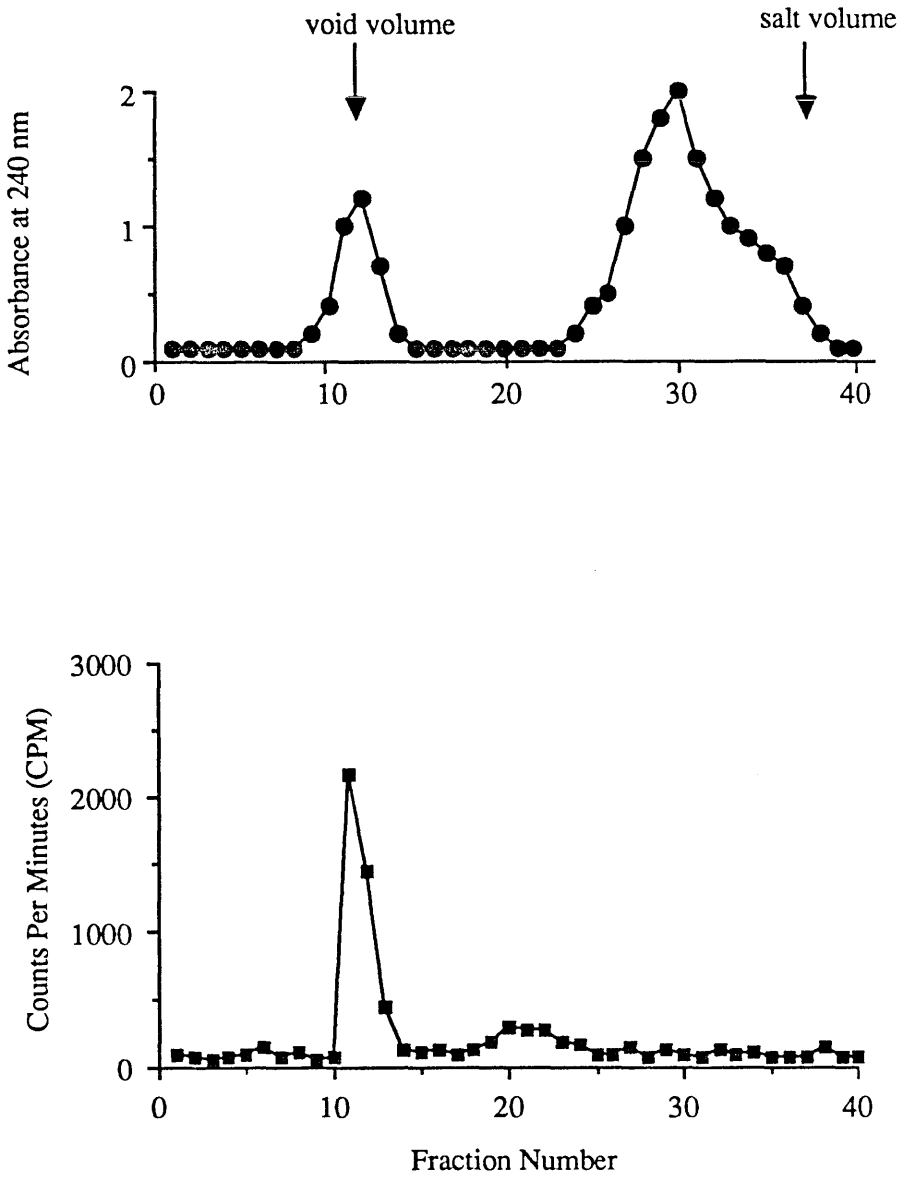


Figure 21: Preparation of the immunogen showing the incorporation of radioactive tracer into the void volume protein peak

three-way stop-cock. One of the syringes was filled with the adjuvant and the other with an equal volume of immunogen in solution. The aqueous solution was passed rapidly into the oil and the resulting mixture was passed backwards and forwards between the syringes until the required viscosity had been achieved. The emulsion was tested to determine its viscosity by allowing a few drops of the mixture to fall into a beaker of cold water. If the emulsion is of the required viscosity, then the drops remain intact on the surface of the water. This response indicates that the water phase containing the immunogen is entirely enclosed within the oil and a successful emulsification has been achieved.

The emulsion was injected into the peritoneal cavity of the mice. The animals were boosted 14 days later with 100µg of the immunogen emulsified with incomplete Freund's adjuvant (without the heat-killed mycobacteria) intra-peritoneally (I.P.). This was followed two weeks later by an I.P. boost of 100µg of the immunogen alone in saline,and four days later the animals were sacrificed and their spleens removed to carry out cell fusion, as outlined below. Table 7 summarises the immunisation schedules.

3.2.2. Cell culture media

3.2.2.1. Cell maintenance medium

The medium used for feeding and maintaining cells in culture was termed "maintenance medium". All media used in this work were obtained from Flow Laboratories, U.K. RPMI-1640 x 10 was used as the basic medium. It was prepared as follows:-

To 170ml sterile, distilled water add

20ml 10 x RPMI-1640

2ml L-glutamine (200mM)

2ml penicillin/streptomycin (10000 iu/ml)

2.5ml sodium pyruvate (11mg/ml)

6.5ml 7.5% sodium bicarbonate solution

20ml foetal calf serum (heat inactivated at 56^{o}C for 30 min)

	Number of days	Injection medium	Amount of immunogen	Site of injection
First injection	1	Complete adjuvant	100 µg	Intra-peritoneal (I.P.)
Second injection	14	Incomplete adjuvant	100 µg	I.P.
Third Injection	28	Saline	100 µg	I.P., Intra-venous.
Fusion	32	___	___	___

Table 7: Immunisation schedule for the production of monoclonal antibodies

3.2.2.2. Cell fusion medium

This medium was only used during fusion procedures since it is serum-free.

To 170ml of sterile distilled water the following reagents were added :-

20ml 10 x RPMI-1640

2ml L-glutamine

2.5ml sodium pyruvate

2ml penicillin/streptomycin

6.5ml Hepes buffer

3.2.2.3. Hybridoma selection medium (HAT)

To the above maintenance medium (200ml), 2ml of hypoxanthine(H) aminopterin(A) and thymidine(T) obtained as 50 x concentrated solution was added. HAT and HT (also obtained at 50 x concentration) were frozen in 2ml aliquots and added to the medium when required.

3.2.2.4. Storage (freezing) medium

This was made up immediately before use with foetal calf serum containing 10% (v/v) of dimethylsulphoxide, and was kept on ice.

3.2.3. Polyethylene glycol 1500 (PEG) solution

PEG (1g/fusion) was weighed out and melted by autoclaving at 12 pounds per square inch. for 15 minutes. 1ml of Hepes buffered fusion medium was added to the now liquid PEG. This prepared PEG was stored at 4°C.

3.2.4. Phosphate buffered saline (PBS)

This buffer helps to prevent the spleen cells from clumping during teasing. It was prepared as follows:-

To 1 litre of distilled water was added

2g KH_2PO_4

8.56g Na_2HPO_4

4.5g NaCl

The pH of the buffer was adjusted to 7.4 and autoclaved.

3.2.5. Materials for Screening Procedure

3.2.5.1. Coating buffer

The coating buffer that was used was prepared as before (see Plate-coating, page 74)

3.2.5.2. Glazing (blocking) buffer

0.1 M phosphate buffer pH 7.4 containing 1% (w/v) BSA

3.2.5.3. Washing buffer

0.01M phosphate buffer pH 7.4 containing:

0.15 M sodium chloride

0.5% (w/v) BSA

0.1% (v/v) Tween -20

3.2.5.4. Assay buffer

The assay buffer for ELISA and RIA procedures to screen for the monoclonal antibodies was the same as the washing buffer prepared as above.

3.2.5.5. Plate Development Buffer

Citrate/phosphate buffer 0.05 M pH 5 containing 0.007% (v/v) hydrogen peroxide

3.2.5.6.Peroxidase labelled rabbit-anti mouse and swine anti-rabbit antibody

The above antibodies were obtained from Sigma, U.K. and used at a 1:1000 dilution.

3.2.5.7. Enzyme substrate (obtained from Sigma)

O-phenylenediamine (25 mg in 50 ml of citrate buffer)

3.2.5.8. Stopping solution

Concentrated sulphuric acid (12.5% v/v).

3.2.6. Cell Fusion

3.2.6.1. Preparation of cell suspensions

3.2.6.1.1. Peritoneal macrophages (feeder cells)

Macrophages have been shown to greatly improve the yield of hybridomas in fusion cultures (Fazekas de St. Groth and Scheidegger, 1980). They clear the fusion cultures of dead parental cell debris by the end of the first week and serve as a feeder layer. They also provide a substantial cell density to facilitate the growth of emerging hybridomas and survive until the end of the experiment.

Macrophages were obtained from normal adult Balb/C mice as follows:- the abdominal wall was swabbed with 70% ethanol. A small flap of skin was lifted in the middle of the abdomen and snipped off. The cut was extended forwards and backwards with great care to ensure that the peritoneum was not punctured. Using a 25G needle, 5ml of cold maintenance medium was injected into the peritoneal cavity. The abdomen was gently palpated and the medium was withdrawn using a 21G needle into the same syringe, making sure that the bevelled edge of the needle was pointing outwards. Usually one mouse yielded approximately 2.5 x 10^6 macrophages. They were always preseeded in microtitre plates on the day prior to fusion in 10^4 cells/100µl/well.

3.2.6.1.2. Spleen cells from immunised animals

Four days after the last injection, the animals were sacrificed and their spleens removed aseptically and placed in a sterile petridish containing 10ml of PBS-J. After removing the adherent fat from the spleen, it was teased using 21G needles to release the cells. As the medium became cloudy it was pipetted off into a 10ml centrifuge tube. Fresh medium was added until the whole organ had been broken down.

The tube was left on a rack to allow the debris to sediment. The supernatant was transferred to another tube, centrifuged at 200g to sediment the cells which were then washed twice in serum-free medium.

3.2.6.1.3. Myeloma cell line

Originally, the pioneers of the fusion procedure (Kohler and Milstein, 1975) used immunoglobulin secreting myeloma cells X63-Ag8 (which secreted IgG_1, k) and MPC-11 (which secretes IgG_{2b}, k). Variants of these cell lines have since been selected producing only light chain immunoglobulin or none at all. In this study, an Ag8 cell line was used which was kindly provided by Dr. M. Ritter (Department of Immunology, RPMS). The cells were usually stored in liquid nitrogen in the freezing medium and thawed rapidly in a water bath at 37°C prior to use. Maintenance medium (5ml) was added to the tube a drop at a time over a period of 5 minutes whilst the tube was being shaken. The cells were then washed once in the maintenance medium and placed into a 25ml tissue culture flask. The cells were split the day before the fusion, and resuspended in flasks at 3×10^5 cells/ml in order that they would be at an exponential phase of growth and ready for fusion on the following day.

3.2.6.2. The fusion

Sendai virus was originally used as a fusogen but has now been totally replaced by polyethylene glycol (PEG) which destroys the surface tension forces that normally cause cells to repel each other and thus allows the membranes to fuse.

Myeloma cell suspension containing approximately 10^7 cells and spleen cell suspension containing approximately 10^8 cells were pelleted in a 50ml tube by centrifugation at 200g for 10 minutes. The two pellets were resuspended, pooled in fusion medium and centrifuged again at 200g for 10 minutes. The supernatant was removed and the pellet resuspended by gently tapping the tube. The tube containing the cells was placed into a beaker of water at 37°C. Warm PEG (1ml) solution was added slowly to cells over a period of 1 minute with constant stirring of the mixture. Immediately 1ml of fusion medium was added and stirred gently over one minute. Further medium (4ml) was added over 3 - 4 minutes without stirring. The tube was gently inverted to mix the contents.

Maintenance medium (20ml) and maintenance medium (20ml) containing 15% foetal calf serum was added to the fusion mixture and incubated at 37°C for 1 - 2 hours.

The hybrids were spun down and resuspended to 2.5 x 10^5 cells/ml. The hybrids were plated out in 24-well plates at 1ml/well into 3 plates already preseeded with macrophages. A control well was included in all the fusions in order to monitor the efficiency of the HAT selection.This consisted of one well with only myeloma cells and macrophages.The plates were then incubated at 37°C.

3.2.6.3. Hypoxanthine, Aminopterin, Thymidine (HAT) selection

The unfused myeloma cells grow faster than the hybridomas, and would rapidly outgrow them. Therefore, the mechanism of self-destruction is operated via "HAT" selection. This involves changing the composition of the tissue culture medium (see figure 22). The cell line used for fusion had been obtained by growing the myeloma cells in the presence of a drug called 8-Azaguanine. The myeloma cells are normally killed by azaguanine but some became resistant to the effects of this drug by switching off permanently the production of the enzyme hypoxanthine phosphoribosyl transferase (HPRT). The cells are known to be HPRT negative after this selection. When such cells are grown in a mixture of HAT medium, the cells will die because they can no longer synthesise DNA. The aminopterin blocks the main pathway for purine and pyrimidine synthesis, and therefore the production of DNA. Normal cells which possess HPRT can utilise the hypoxanthine to make purines and thymidine to make pyrimidines but the HPRT-negative cells cannot do this and they die in culture. The normal lymphocytes from the immunised animals' spleen cannot grow in culture, therefore, they die anyway. The parent myeloma lacking HPRT will not grow in the HAT selection medium. Therefore the only surviving cells are the hybridomas.

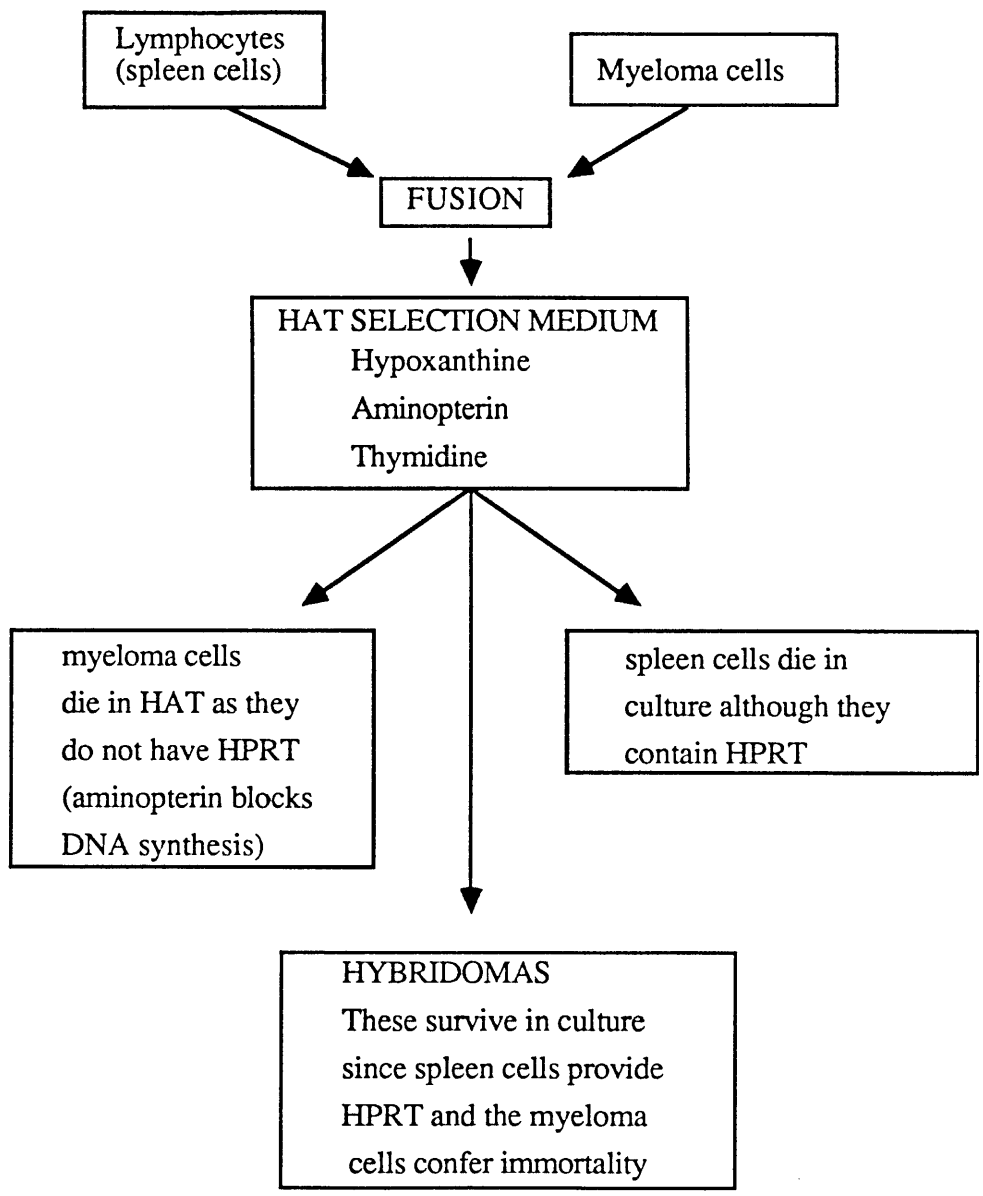


Figure 22: Diagram showing the HAT selection procedure

3.2.6.4. Maintenance of fusion cultures

Hybridoma cultures were incubated at 37°C in a humidified incubator with 5% carbondioxide and were fed by removing 1ml of culture medium and replenishing with 1ml of fresh medium twice weekly after the third week.

3.2.6.5. Screening and expansion of fusion cultures

The choice of assays used during the screening stages to detect and clone a hybrid secreting the desired antibody is very important and differs from testing a polyclonal antisera. The important considerations are firstly, that the concentration of the antibody in the culture fluid is usually lower than that of a hyperimmune serum and secondly, traditional immunoassays often rely on polyvalent recognition of antigens typically obtained with polyclonal antisera. Three possible screening strategies were examined as outlined below.

3.2.6.6. Binding assays

a) Insolubilization of calcitonin and CGRP (ELISA method)

In this type of screening assay, the antigen i.e. hCT or hCGRP is insolubilised on to plastic microtitre plates or polystyrene balls. The antibody in the culture fluid is then allowed to react on the coated plates. The amount of antibody bound to the plate is measured by binding of a second labelled antibody capable of recognising the first antibody. The second antibody can be labelled in several ways, e.g. with a radiolabel or an enzyme. The procedure used in the present study is detailed below.

Ninety-six well microtitre plates were coated with synthetic hCT and hCGRP (0.1μg/well) in a 0.1 M phosphate buffer pH 7.4 and left overnight at 4°C Unoccupied binding sites were then blocked by overnight incubation of the plates with 0.1M PBS containing 1% BSA pH 7.4 at 4°C. The plates were then washed four times with a washing buffer of 0.01M phosphate buffered saline (pH 7.4). Culture fluids from the rapidly growing hybridoma wells were placed

on the coated plates and incubated at 37°C for 1 hour. The plate was washed four times with a washing buffer. A peroxidase labelled anti-mouse antibody was added to the plate at 1:1000 dilution and incubated at 37°C for 1 hour. After a washing step, substrate solution (200 µl, 25 mg O-phenylenediamine in 50 mls of a citrate/phosphate buffer 0.05M, pH 5 containing 0.007% hydrogen peroxide) was added and incubated at room temperature for 30 minutes. Sulphuric acid (50 µl/ well; 12.5% v/v) was added to all the wells to stop the enzyme reaction and intensify the colour change from yellow to brown. The absorbance at 492nm was measured using the Titertek Multiskan ELISA plate reader (Flow Labs, England). The positive wells were those which had an absorbance value of at least 0.5 (after subtracting the blank reading).

b) Direct binding assay

In this type of assay, the anti-mouse antibody was first bound to the microtitre removal wells. The spent culture fluid was then added so that any antibody present can bind on to the first antibody. ^{125}I-labelled hCGRP and hCT (50 µl/well, 10,000 counts/100 seconds) are then added and incubated. The wells were then washed and counted in the γ-counter. The positive wells were those which showed at least 20% specific binding.

c) Indirect binding assay (RIA)

In this assay, the antibody in culture fluid is added in a tube to the labelled hCGRP or hCT. After incubation, the separation of bound/free fractions is carried out using charcoal suspension. The label is then counted in the free fraction and the results are inversely proportional to the signal generated, i.e. high counts signifies minimum binding

3.2.6.7. Cloning

The timing of cloning is the most important factor after the fusion. The positive wells must be cloned as soon as possible since multiple clones in a single culture compete for growth and this, together with chromosome segregation, conspires against stability of expression. There are three techniques

available for cloning. The first is called soft agar cloning where the cells from the positive wells are grown on a semi-solid surface. The agar stops the cells moving so that it is easy to see single colonies of cells as little clumps. The cells are then removed from the agar and subcultured.

The second method is known as single cell micromanipulation, where the cells are picked up by an inverted microscope and a drawn out pipette and plated out.

The third and the most common method is called cloning by limiting dilution, where the cells are diluted out in the wells of a 96 well microtitre plate until diluted down to a theoretical 0.3 cells per well (after the second and third cloning). The antibody obtained from the wells should be the result of a single hybrid clone.

Of the three methods, the limiting dilution method was the easiest and the quickest, but the single cell manipulation is probably the most accurate and needs to be performed only once. Cloning by limiting dilution was used in this work.

3.2.6.8. Cryopreservation of cells

This procedure enables the preservation of the parental myeloma cell line as well as the established hybridomas. However, freezing of the cells which were not cloned after the primary fusion was not always successful, presumably due to overgrowth by non-producing cells. The basic freezing procedure was as follows:- the cells were centrifuged and resuspended at 5 - 10 x 10^6 cells per ml in 90% FCS and 10% DMSO. Aliquots (1.5ml) of this suspension were then pipetted into plastic storage ampoules and frozen slowly at -70°C in a plastic container placed in a polystyrene box for at least 12 hours. The ampoules were then transferred into liquid nitrogen. The recommended freezing rate was 1°C per minute. A procedure for freezing cells in tissue culture plates has been reported (Wells and Price, 1983).

In contrast, thawing was done speedily. The ampoule was thawed in a 37°C water bath and the contents were transferred to a small plastic flask containing

10ml of the maintenance medium. Only 10 - 20% of the cells were recovered from freezing.

3.2.7. Antibody production

3.2.7.1. Expansion of hybridomas *in vitro*

The hybridomas were maintained in culture for the large scale production of antibody in animals and also to maintain the stock of hybridomas. Typical culture supernatants yield upto 100μg/ml of antibody depending upon the cell density and rate of growth. Cultures *in vitro* provide a more pure preparation of antibody. The only protein contaminants were from the FCS, which has up to 1.3µg/ml basic immunoglobulins.

3.2.7.2. Expansion of hybridoma *in vivo*

Mice primed with mineral oil tend to be more susceptible to the development of spontaneous mineral oil induced plasmocytomas (MOPCs) and this fact is used to encourage hybridoma growth *in vivo* (Hoogenrad, Helman and Hoogenrad, 1983; Gillette, 1987).

The mice were primed by intraperitoneal injection of 0.5 ml pristane (tetramethyl pentadecane), (Sigma) 5-10 days before intraperitoneal inoculation with 10^6 to 10^7 hybridoma cells. Hoogenrad *et al.* (1983) have shown that a 10 day interval between pristane priming and tumor injection gives a higher percentage of responding mice. However, the rate of growth of the resulting ascites tumors was variable and was from less than two to more than five weeks. Sterile collection of the ascitic fluid was performed as follows. The anaesthetised mouse was placed on its back and a 21G needle was inserted into the peritoneal cavity. The fluid was then withdrawn slowly into the syringe.

Ascitic fluid (10ml) was regularly obtained from a mouse. Ascites fluid will be contaminated with mouse immunoglobulins. However, other protein contaminants are readily removed by ammonium sulphate precipitation.

Purification of monoclonal antibodies was not necessary for the purpose of developing an immunoassay and was therefore not carried out.

3.2.8. Characterisation of monoclonal antibodies

The characterisation of monoclonal antibody involves the determination of antibody class, antibody affinity and epitope analysis.

3.2.8.1. Determination of antibody class

The antibody class was determined by a recent method known as direct detection of antibody. It involves the adhesion of antibody secreting hybridoma cells on to a glass slide (cytospins) followed by fixing and mounting procedures. The slides are then incubated with anti-isotype antibodies labelled with a fluorescent dye. After incubation and washing steps, the slides are observed under a UV microscope to locate the monospecific antibody secreting cells. The procedure is as follows:- the hybridomas cells were harvested, washed once and resuspended to approximately 10^6 cells/ml. 10% FCS was added to the cell suspension as this improved cell adhesion. Six cytospins for each cell line were prepared using 100µl of the cell suspension per slide.

The slides were then fixed for one minute in methanol and then transferred to PBS. Dilutions (1:20) of the anti-isotype antisera (mouse anti-IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM purchased from Sigma) were prepared. 50µl of each anti-isotype to the first five slides were added and 50µl of PBS to the sixth slide as a negative control and incubated for 45 minutes in a moist box.

The slides were washed three times in PBS, allowing 3 minutes per wash. The slides were then dried very carefully around each cytospin and 50µl of 1:5 dilution of anti-mouse immunoglobulins labelled with fluorescein isothiocyanate (FITC) was added and incubated for 45 minutes. The slides were then washed three times as before, dried and mounted in 'hydromount'. When viewed under a UV microscope, a positive cytospin was observed for IgG_1 and IgG_{2a} as a dark nucleus and bright green cytoplasm (see plates 1, 2, 3, and 4).

Plate1. Cytospin preparation of the anti-calcitonin antibody producing hydbridoma (CT65B8) to characterise the antibody isotype.

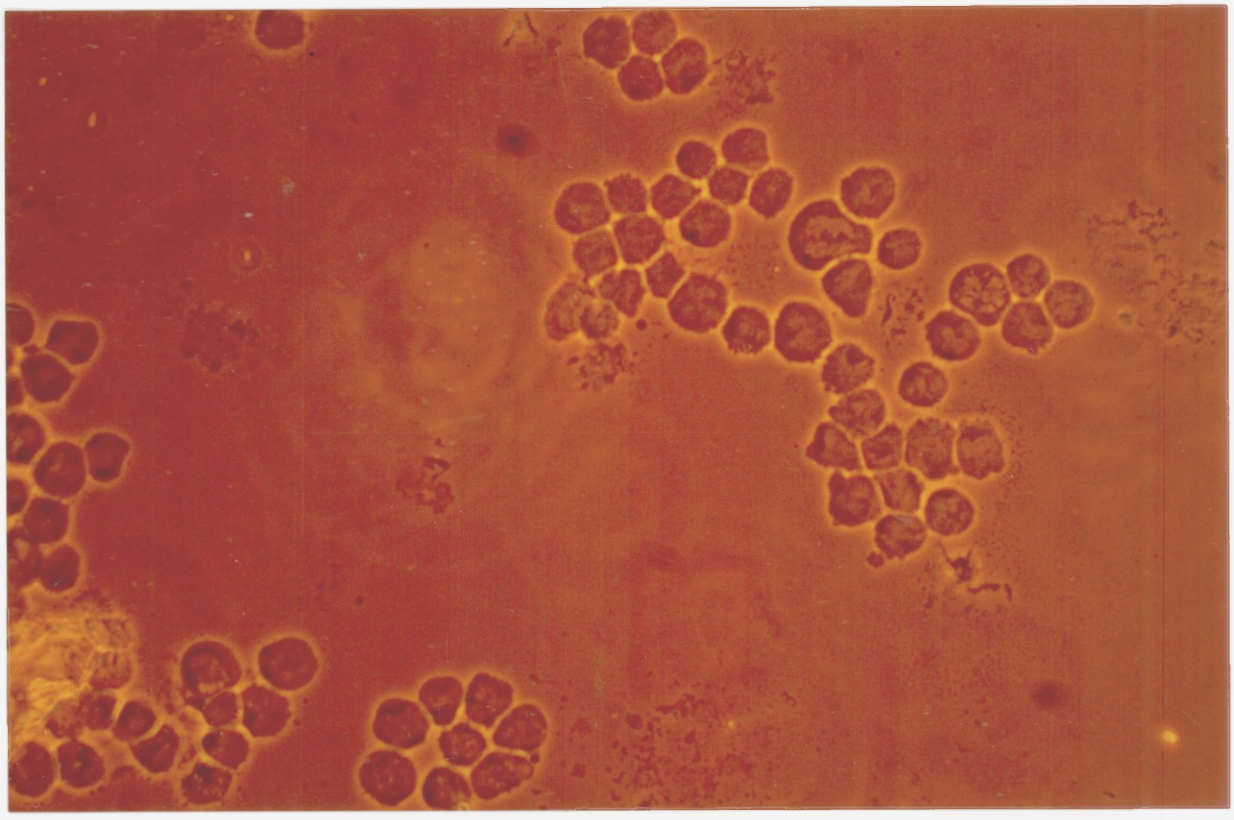


Plate 2. The same preparation of the cells viewed under the UV microscope. The positive cells are characterised by the fluorescent cytoplasma. These cells were specific for IgG_1 class of antibody.

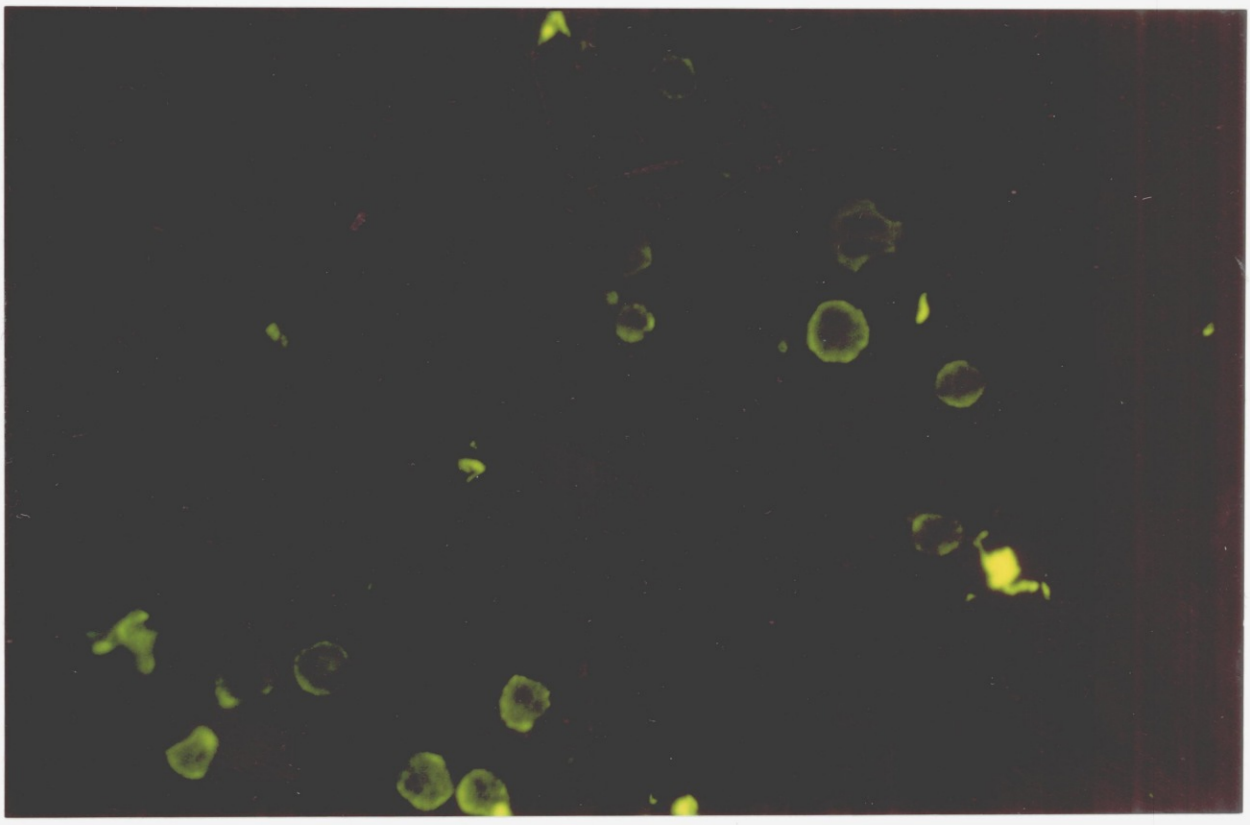


Plate 3. Cytospin preparation of the antibody producing cells to show the comparison of the negative anti-isotype antibody under the light microscope.

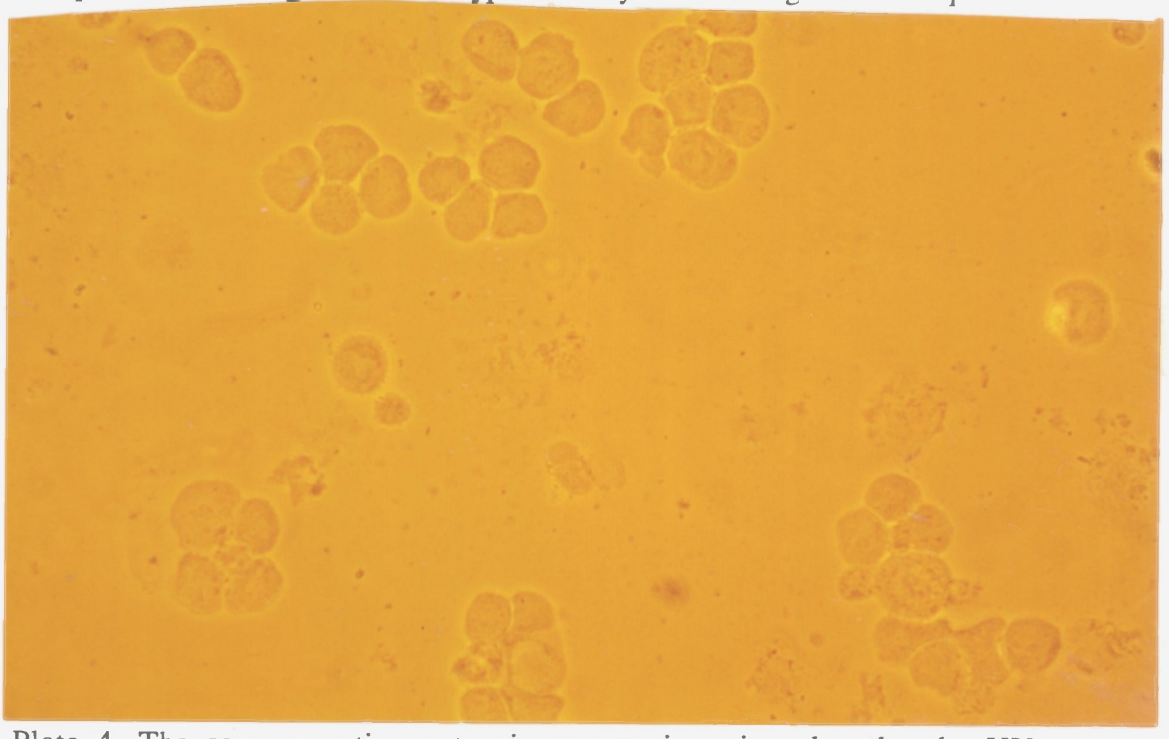
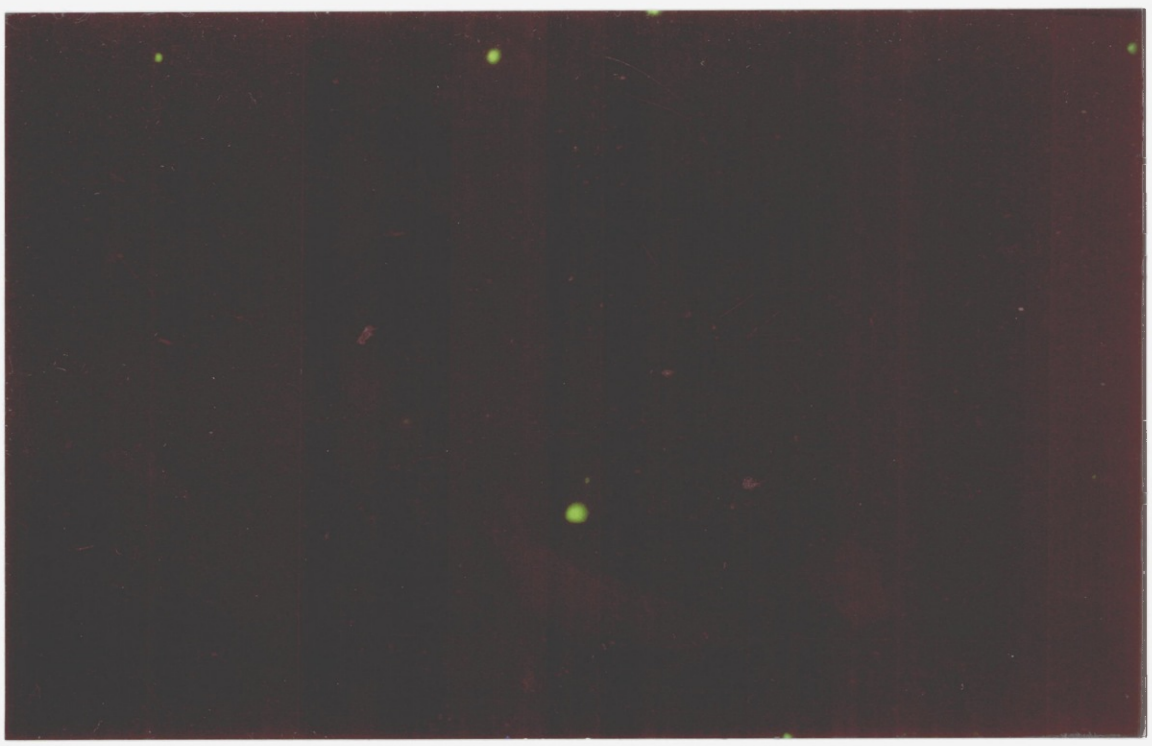


Plate 4. The same negative cytospin preparation viewed under the UV microscope. Note the shadow of the cells without any fluorescent staining.



3.2.8.2. Determination of antibody titres

Titres of monoclonal antibodies CT/65/B8 and CGRP/H3 were determined by both the ELISA and RIA. The titres were compared with those of the corresponding polysera OC31 (for hCT) and CC21 (for CGRP). 96- Well microtitre plates were coated overnight with synthetic calcitonin and CGRP (0.1µg/well) in a 0.1 M phosphate buffer pH7.4 at 4°C. Unoccupied binding sites were then blocked by overnight incubation of the plates with 0.1M PBS containing 1% BSA pH7.4 at 4°C. The plates were then washed with a washing buffer of 0.01M phosphate buffered saline (pH7.4) four times.

The plates were now ready for use. The antibodies were diluted 1: 5,1:10, 1:50,1:250, 1:1250,1:6250, 1:31250 in a 0.01 M PBS pH 7.4 (0.15M sodium Chloride, 1% BSA, 0.1% Tween 20) and incubated in the plates for 1 hour at 37°C. The plates were then washed three times with a washing buffer. The reaction was revealed with a peroxidase labelled rabbit anti-mouse (100µl) and swine anti-rabbit antibody placed on the plate at 1:1000 dilution in each well and incubated for a further 1 hour at 37°C. After a washing step, 200µl of the substrate O-phenylenediamine was placed in each well as before and incubated for 30 minutes at room temperature in the dark. Sulphuric acid (50µl, 12.5% v/v) was added to all the wells. Absorbance was measured at 492nm on a Titertek Multiskan ELISA reader (Flow Laboratories, England). A dilution which gave an O.D. of 1 was the working dilution and therefore the titre of the antibody. CT/65/B8 was used at a dilution of 1:1250 for epitope mapping.

The titres of the monoclonal antibodies were determined by both the ELISA method and the RIA. (The affinity constants of these monoclonal antibodies can be determined by the method of Van Heyningen, Brock and Van Heyningen (1983) but was not carried out since the titre was so low.)

3.2.8.3. Determination of specificity

The specificity of the anti-calcitonin monoclonal antibody was determined by a competitive inhibition ELISA assay which is outlined below in order to identify the epitope recognition site. This work was carried out at the Institut Gustave-Roussy, Paris, France in collaboration with Professor C. Bohuon, Professor D. Bellet and Dr. P. Motte.

Epitope mapping was carried out using the hybridoma cell line CT/65/B8 which showed the highest titre of anti-calcitonin antibody (see table 9). The antibody was diluted in a buffer and incubated with serial dilutions of synthetic peptides of defined sequences shown in table 11 at 1 x 10^5 to 1 x 10^8M/l final concentration for 18 hours at 4°C. The unreacted anti-hCT activity was measured by ELISA with plates coated at 0.1µg hCT per well. In each assay, the following controls were employed:

a) monoclonal antibody pre-incubated with buffer [total binding] and

b) monoclonal antibody directed against an unrelated antigen [non-specific binding] pre-incubated with each peptide or buffer. Inhibition of monoclonal antibody binding to hCT was calculated according to the following formula:-

$$100 \times 1- \frac{[\text{O.D. of antibody incubated with test peptide}]-[\text{O.D. of non-specific antibody with test peptide}]}{[\text{O.D. of antibody incubated with buffer}]-[\text{O.D.of non specific antibody incubated with buffer}]}$$

The above formula gives the % inhibition of binding of the test peptide with the monoclonal antibody. There was no cross-reaction with other non-specific peptides.

3.3. Results

3.3.1. Cell fusions

In table 8 the results of the HAT selection following one fusion is shown. One day after the fusion, 100% of the cells in the control well (which is the well containing only myeloma cells) appeared to survive after the addition of the HAT X2 medium, but after 7 days, only about 8% of the cells appeared alive. At this time, the surviving hybridoma cells were seen to be replicating. By the fourteenth day after the fusion, there were no surviving myeloma cells in the control well. In approximately 75% of the remaining wells, hybridoma colonies appeared to be growing. Three weeks after the fusion, 90% of the wells had rapidly growing hybridoma colonies which were visible with the naked eye and appeared as large opaque clumps of cells.

It was vital to include a control well in order to be certain that the HAT selection was successful.

3.3.2. Screening and expansion

The choice of assays available for the screening of antibody secreting clones have been discussed (section 3.2.7.). All three methods were used in this study. The first method which involved binding of the antigen (hCT and hCGRP) to a solid phase was the most convenient method in the sense that the coated plates could be prepared and preserved whilst the fusion cultures were undergoing the HAT selection. As soon as the hybrid colonies appeared, they could be screened without any delay. This rapid screening was necessary in order to locate and expand the antibody secreting cells.

The main drawback of this screening method was that large amounts of peptides (100μg/plate) were required for the solid phase binding (recommended coating concentration of 1μg/well). However, it was found that 0.10μg/well was sufficient for one screening assay). This type of screening method can be disadvantageous where the immunising antigen is in short supply.

Days after fusion	Medium	% of wells with colonies appearing	% of surviving cells in control wells(myeloma cells)
1	HAT X 2	-----	100
7	HAT X 1	25	8
14	HT	75	-----
21	culture medium	90	-----

Table 8: HAT selection procedure and the percentage of surviving myeloma cells in the control well of one fusion

Antibody name	Medium	Type	Titres	
			EIA	RIA
OC31(CT)	Serum	Polyclonal	1/2,000	1/30,000
CT/65/B8	Ascites	Monoclonal	1/1,250	1/,500
CC21 (CGRP)	Serum	polyclonal	1/31,000	1/60,000
CGRP /H3	Ascites	Monoclonal	1/30	---
CGRP/D3	Ascites	Monoclonal	1/30	---

Table 9: The titres of the monoclonal antibodies as measured by ELISA and RIA. Titres obtained with corresponding polyclonal antisera are shown here for comparison

The second type of screening method involved binding of anti-mouse antibody to the solid phase (removable microtitre wells) in order to capture any antibody present in the spent culture fluid which was then reacted with a labelled peptide to confirm the results obtained from the first screening method. This method was lengthy compared to the first method and three incubation steps were required. The frequent preparation of ^{125}I-labelled peptides was also required. Hence this method was used only to confirm results obtained from the first screening.

The third method (RIA), which did not involve the use of a solid phase, was performed by incubating the labelled peptides with the spent culture fluid and separating the antibody bound fraction from the free form by means of charcoal. The radioactivity present in the pellet (antibody free fraction) was estimated and an inverse relationship existed (i.e. low counts high binding).

Positive controls, which were high titre rabbit polyclonal antisera, were included in all the screening methods. The titres of the antibodies measured by both the ELISA (method 1) and the RIA (method 3) are compared in table 9 and figure 23. The titres of the rabbit polysera when measured in the RIA were higher than those measured by the EIA whereas the reverse was found to be true for the titres of the monoclonal antibodies. The reason for this discrepancy is unclear but is most likely to be due to the synergistic effects of polyclonal antibodies to form an internal lattice in the liquid phase of the RIA and to the lower affinity for binding on the antigenic sites of the peptides on the solid phase.

3.3.3. Determination of antibody isotype

The method of antibody class determination was by the direct detection of the antibody secreted by the cells into the cytoplasm and in their immediate surroundings by using specific anti-isotype antibodies and visualising the reaction by the addition of anti-mouse antibodies labelled with FITC and locating the positive cells under the UV microscope.

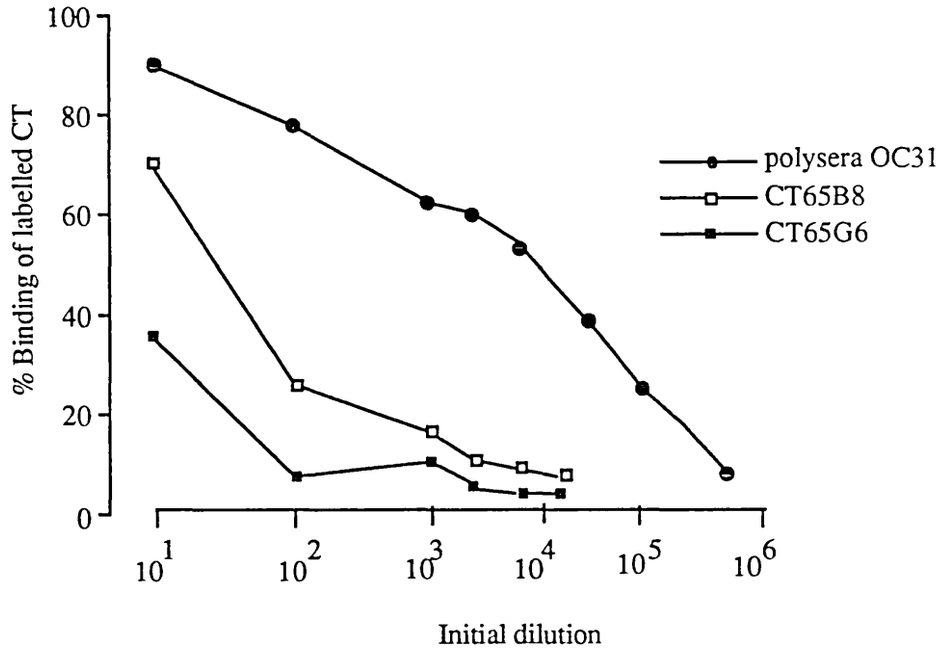


Figure 23: Comparison of the titres obtained for monoclonal antibodies (CT65B8 and CT65G6) with that of the polyclonal (OC31) antibody

Table 10 shows the results obtained for the established cell lines (5 for anti-hCT and 2 for anti-hCGRP). The cells from the original positive well ("mother well") containing polyclonal antibodies were also screened and found to be positive for IgM as well as IgG. Subsequent cloning established the cell lines with monospecific isotype.

The determination of antibody class is very important particularly when a purification step is required; for example, it is useful to know the isotype if the clone selected is secreting an IgM as it will not bind to protein A sepharose. In the present study, the purification step was not performed as the antibody titre was low.

3.3.4. Epitope analysis

Table 11 lists the sub-peptides of hCT used for epitope analysis. The competitive ELISA method was used for epitope mapping of CT/65/B8 using the titres listed in table 9. The inhibition profiles (see figure 24) confirmed that the antigenic determinant was located in the 28-32 amino acid region, since a subpeptide of sequence 28-32 with a proline amide inhibited the binding of the monoclonal antibody to hCT. The resulting curve was parallel to that observed for 1-32 hCT. The sub-peptide 26-32 acid substitute did not bind to the antibody.

Monoclonal Antibody	Antigen	Antibody class
CT 65 B8	hCT	IgG1
CT 65 G6		IgG1
CT 65 G4		IgG1 (weakly +)
CT 65 D11		IgG1, IgM
CT 65 poly		IgG1, IgM
CT 65 A11		IgG1 (weakly +)
CGRP 25/A/C5/H3	hCGRP	IgG2a
CGRP 72/A/89/D5		IgG1
CGRP 72 poly		IgG1, IgM

Table 10: Isotype mapping of monoclonal antibodies

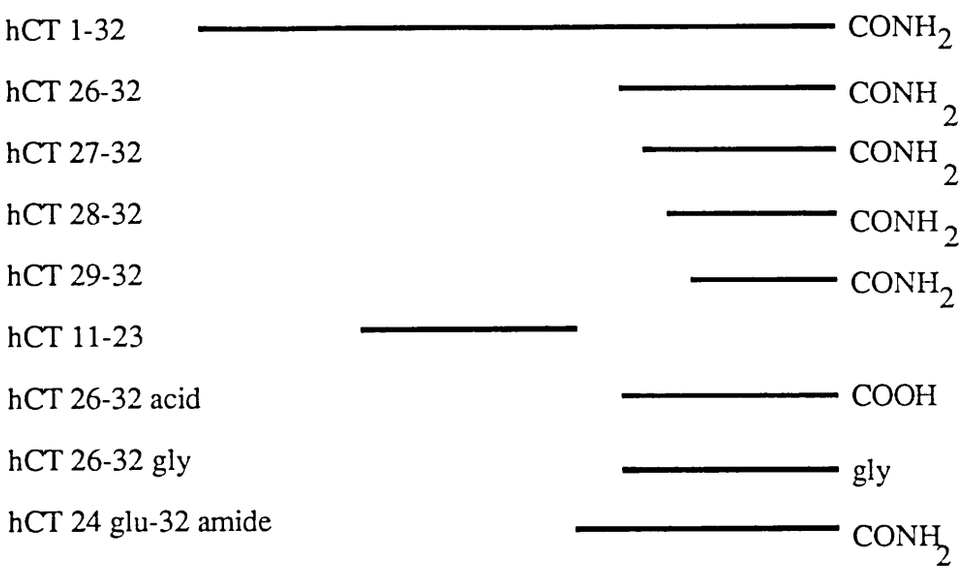


Table 11: Peptides and sub-peptides used for epitope mapping

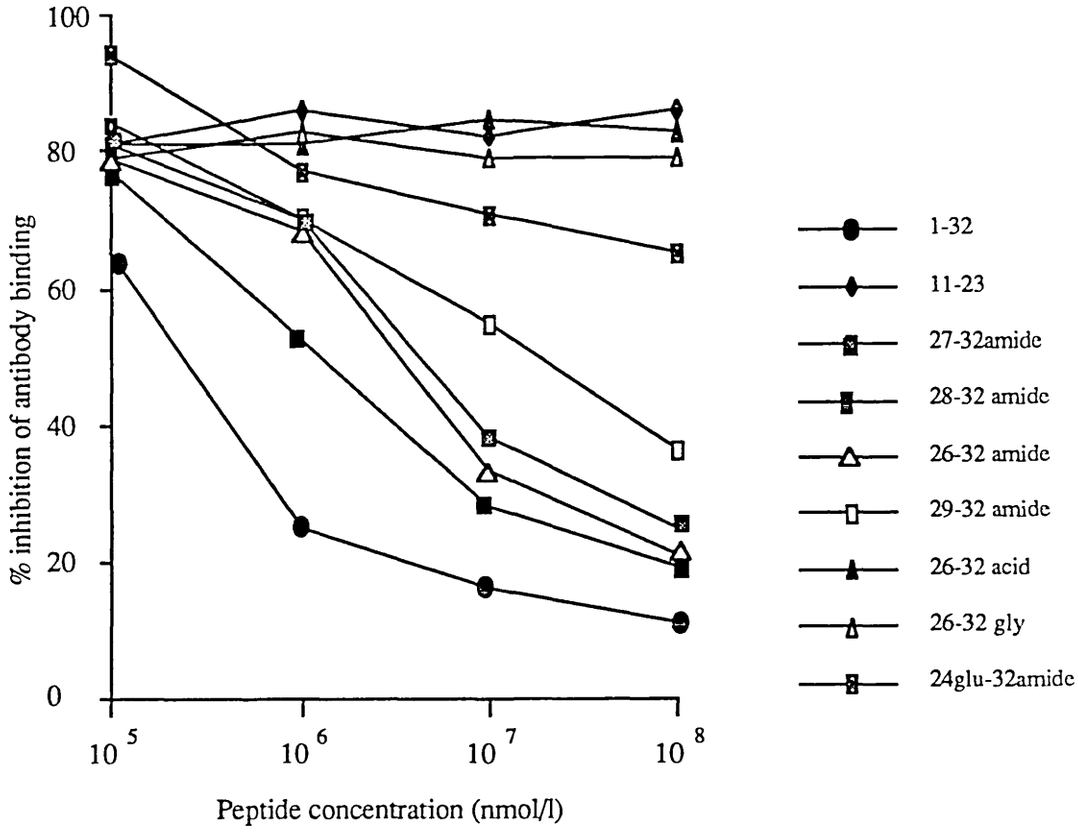


Figure 24: Epitope analysis (mapping) of the monoclonal antibody CT65B8 with hCT peptide and subpeptides

3.4. Discussion

The monoclonal antibody production technique has become popular in recent years and has enabled the vast production of monospecific antibodies. The production of monoclonal antibodies is more demanding in terms of time, effort and money than simple immunisation to obtain polyclonal antisera.

The outcome of a fusion experiment is not only dependent on factors such as the amount of immunogen, type of animal species and peptide-carrier strategy, but also on the structures of the peptides in question. Some epitopes in polypeptides may be conformational, consisting of amino-acid residues from different parts of the polypeptide chain which are brought together into proximity in the native tertiary structure. In other cases, epitopes present in intact molecules may also be identified in short peptides derived from the molecule. This observation provides a method of analysis of antigenic regions where a complete amino acid sequence is known and various fragments are available.

The monoclonal antibodies against hCT raised in the present programme of work have been studied from this point of view. This information can be used to analyse the relative orientation of epitopes in the native protein structure, for example, in the identification of peptide sequences which are exposed at turning points of the hCT molecule. The results suggest that although hCT has a molecular weight of only 3,500 it has several antigenic determinants exposed on its surface. This would agree with the three-dimensional model for CT proposed by Moe, Miller and Kaiser (1983) and Moe and Kaiser (1985) which describes three structural regions in the molecule; a loop involving the N-terminal disulphide bridge, an amphiphilic alpha-helic segment between residues 8-22 and a hydrophilic random coil sequence from residue 23-32. A disulphide bridge links aminoacids 1 and 7. Four out of seven amino acid residues in this region are hydrophobic (lys, gly, asn, leu) and this sequence is engaged in a ring of constrained conformation (Granier, Osganthorpe and Robinson, 1978). This conjunction defines a region of poor immunogenic potential (Hopp and Wood, 1981; Palfreyman, Aitcheson and Taylor, 1984), and the production of

monoclonal antibodies directed against 1-10 amino acid sequence of hCT has not yet been reported. In contrast, several authors have described polyclonal antisera against hCT which recognise epitopes present on the 11-32 region of hCT. This region has been typically described as antigenic (Catherwood and Deftos, 1983) and supports the theory that the pentapeptide conformation within a protein is highly dependent on the sequence context (Kabasch, and Sander, 1984; Wilson, Haft, Getzoff, Tainer, Lernet and Brenner, 1985). Proline-containing sequences have been shown to preferentially adopt a beta folding of the protein and this makes them highly antigenic (Schulz-Gahmen, Prinz, Matter, Beyreuther, 1985).

The monoclonal antibody CT/65/B8 produced in the present study recognises this random hydrophilic coil sequence from residues 28-32. The presence of the amidated proline residue was required for immunological activity since substitution of the proline amide with an acid group (as in 26-32 acid or 26-32 gly) showed no binding of antibody during epitope mapping experiments.

As mentioned before, the peptide-carrier strategy may affect the specificity of the resulting antibodies. In a recent study, monoclonal antibodies raised against an N-terminal conjugated octapeptide recognising the C-terminal extremity of a peptide has been reported (Nussberger, Matsueda, Re and Haber, 1985). In the present study, where the immunogen consisted of hCT conjugated by lysine 18 to ovalbumin, the C-terminal proline amide of hCT may be of major importance in the recognition of the 28-32 epitope by the CT/65/B8 monoclonal antibody.

This may also explain the observations of Motte, Ait-Abdellah, Alberici, *et al.*, (1987) that the absence of the proline amide in the pre-prohormone (synthetic peptide PQN-37 and PTN-47) dramatically decreased their recognition by the monoclonal antibody which was specific for the epitope located in the 26-32 amino acid region of hCT.

The titres of the monoclonal antibodies raised in this study are very low relative to their polyclonal counterparts. As a result, these antibodies cannot be used to produce immunometric assays with the required sensitivity. This preponderance of low affinity of monoclonal antibodies in part reflects the

distribution of affinities seen *in vivo*,and even in polyclonal antisera of apparent high affinity the effective antibody may represent only a small proportion of total binding activity. However, low affinity antibodies do have their uses, particularly in immunohistochemical work, and are more suitable than their high affinity counterparts for use in immunopurification schemes because of the relative ease of elution of antigen in an active form.

The titre of hCGRP monoclonal antibodies was too low for epitope mapping to be attempted. Apart from this work, the production of monoclonal antibodies against hCGRP have not yet been reported. This might reflect in part that the tertiary folding of the molecule is significantly different from that of hCT.

Monoclonal antibodies may be obtained for unique epitopes on a given antigen, whereas polyclonal antibodies bind to multiple sites on an antigen, and occur as a complex mixture which is difficult to resolve except by repeated adsorption or affinity purification with potential cross-reactants or fragments of antigens (Atassi, 1986). In this respect, the fine specificity of monoclonal antibodies is a very important factor, particularly when used in an immunoassay to measure a particular peptide in the presence of potentially cross-reacting degradation products (fragments) or naturally occuring precursors with common epitopes. However, the most useful application of monoclonal antibodies is in establishing immunometric assay formats whereby the specificity of an immunoassay is vastly improved. For this use, pairs of monoclonal antibodies are identified which are able to bind simultaneously to spatially distinct epitopes on a given antigen/analyte without steric hindrance. As the analyte to be detected must bind to two antibodies, each reacting with a different and distinct epitope, the specificity of an immunometric assay is vastly increased.

Recently, two monoclonal antibodies against hCT have been raised for this purpose (Motte *et al.*, 1987). One antibody, designated CT07, was specific for an epitope located in the 26-32 amino acid region along the hCT molecule with a proline amide residue. The second antibody, designated CT08, recognised an epitope located in the 11-17 amino-acid region along the molecule and therefore bound equally not only to the 1-32 hCT molecule, but also to the hCT molecule

engaged in a precursor form. These antibodies should enable us to investigate the pathway of processing of hCT *in vivo*, and the nature of circulating pre-prohormones in human sera. Previous polyclonal antisera raised against hCT have lacked the necessary fine specificity for such studies to be attempted.

These antibodies, when used in an immunometric assay format, selectively recognised circulating molecular species of hCT (1-32 with a proline amide residue which is only found in the 'mature' form of the hormone) and may contribute to the distinction between monomeric CT, the biosynthetic precursors and circulating fragments.

The development and application of an immunoassay for hCT based on monoclonal antibodies is described in chapter 4.

Chapter 4: The development and application of a monoclonal antibody-based immunometric assay for hCT

Summary

A monoclonal antibody-based enzyme immunometric assay was developed for calcitonin in collaboration with Dr P. Motte of The Institut Gustave-Roussy, France. A two-site IRMA utilising these antibodies has already been reported (Motte et al., 1987). In this chapter, the development and application of the assay in conjunction with the method of enzyme amplification is presented. The two monoclonal antibodies employed were specific for hCT and recognised a different and distinct epitope along the molecule. The antibody adsorbed on the solid-phase recognised an epitope located in the 26-32 amino acid region of the hCT molecule. It was particularly specific for the presence of an amidated proline residue at position 32 for immunological recognition. The other monoclonal antibody was conjugated to alkaline phosphatase and recognised an epitope located in the 11-17 amino acid region of the hCT molecule.

This strategy resulted in an assay whereby only the 'mature' form of the hormone could be detected. This precluded the measurement of any precursors (pro-and preprohormone) and fragments which interfere in normal RIA's.The resulting EIA was more sensitive than the corresponding IRMA with the added advantage of using a non-isotopic label.

Human CT levels were measured by EIA as well as the polyclonal antibody-based RIA in the plasma of normal volunteers, MTC patients and post-menopausal women. Earlier findings of low CT levels in normal women were confirmed in this study. It was found that generally, the levels measured by EIA were lower than those measured by RIA. This was probably due to the greater specificity of the EIA in detecting the monomeric form of hCT.

Gel-filtration studies were carried out to partially characterise the immunochemical forms of hCT in MTC plasma. Biological activities of the fractions were assessed in vitro *on the isolated osteoclast system and the monomeric form was found to be biologically active. The higher molecular form (> 60,000 MW) was biologically active but only after urea dissociation.*

4.1. Introduction

In the field of biomedical research monoclonal antibodies have made a great impact, but have not yet become widely used in immunodiagnostics. This reflects the enormous efforts which are put into obtaining monoclonal antibodies suitable for use in assays, which must satisfy demanding standards of sensitivity and specificity. There is also a tendency to look at monoclonal antibodies merely as alternatives to polyclonal antisera for use in the existing competitive RIA's. When good polyclonal antisera are available it has not seemed worthwhile to produce monoclonal antibodies as simple replacements. For this reason, their production was not attempted earlier for hCT and hCGRP.

The advantages of monoclonal antibodies are many (Hunter and Budd, 1981). An indefinite supply of antibody with constant characteristics is produced. A monospecific antibody can be obtained from impure immunogen. The affinity and the fine specificity are defined and can be selected to suit application. Assays, particularly those of the two-site format resulting from the application of specific monoclonal antibodies, give a high signal/noise ratio and thereby increase the sensitivity of the assays.

The main disadvantages of monoclonal antibodies are that firstly, an enormous effort is needed to produce them. Secondly, they predominantly have low affinity and do not always have high titre (as was the case in the present study ; chapter 3). Nevertheless, it was necessary to develop a two-site assay for hCT and hCGRP in order to remove some of the controversies which existed over the physiological levels of these peptides and to further the knowlegde regarding their biological importance.

The problems which exist with the measurement of hCT and hCGRP are the immunochemical heterogeneity which affects their measurement by one-site polyclonal based RIA's and EIA's, and the very low levels of circulating peptides in the plasma of normal individuals (Gudmundsson, Galante, Woodhouse *et al.*, 1969). The main disadvantage of the existing RIA's are that

long incubation times which are needed to achieve the desired sensitivity. This problem was overcome by using enzyme-labelled peptide so that the method of enzyme amplification could be used to enhance the signal produced (Chapter 2). There was also an added advantage of the less frequent preparation of the labelled peptide. But the main drawback of the new assays was that polyclonal antisera were employed, and so the problems in the quantification of the peptides which had multiple immunochemical forms still existed. It was not known which forms of hCT or hCGRP reacted with the antisera. Also, because of the nature of the antisera, it was difficult to carry out epitope mapping. The present assays are adequate for the diagnosis and management of MTC, but are not suitable for measurement of the very low levels of hCT which occur in post-menopausal women (Stevenson and Whitehead, 1982).

Recently, monoclonal antibodies have been produced for hCT (Sturmer, Jones and Sternick, 1984) and a two-site (immunometric) assay has been developed. In a collaborrative study with Dr. Motte of the Institut Gustave-Roussy in Villejuif, France, it has been possible to develop an immunometric assay in which one of the monoclonal antibodies against hCT has been labelled with an enzyme. The steps involved in the development of this assay and its applications are described in this chapter.

The monoclonal antibodies used in this assay were specific in the sense that each antibody recognised a different and distinct epitope along the hCT molecule. The antibody attached to the microtitre plate recognised an epitope located in the 26-32 region of the hCT molecule (see figure 26). Binding of the analyte to this antibody, not only required the presence of the 26-32 amino-acid sequence, but also the presence of an amidated proline residue on the C-terminal (Motte *et al.*, 1987). The enzyme-labelled monoclonal antibody recognised an epitope located in the 11-17 amino acid region of the hCT molecule. By employing this particular assay format, it seemed reasonable to speculate that only the mature form of the hCT molecule would be recognised, thereby avoiding interference in the assay by the precursor molecule which does not

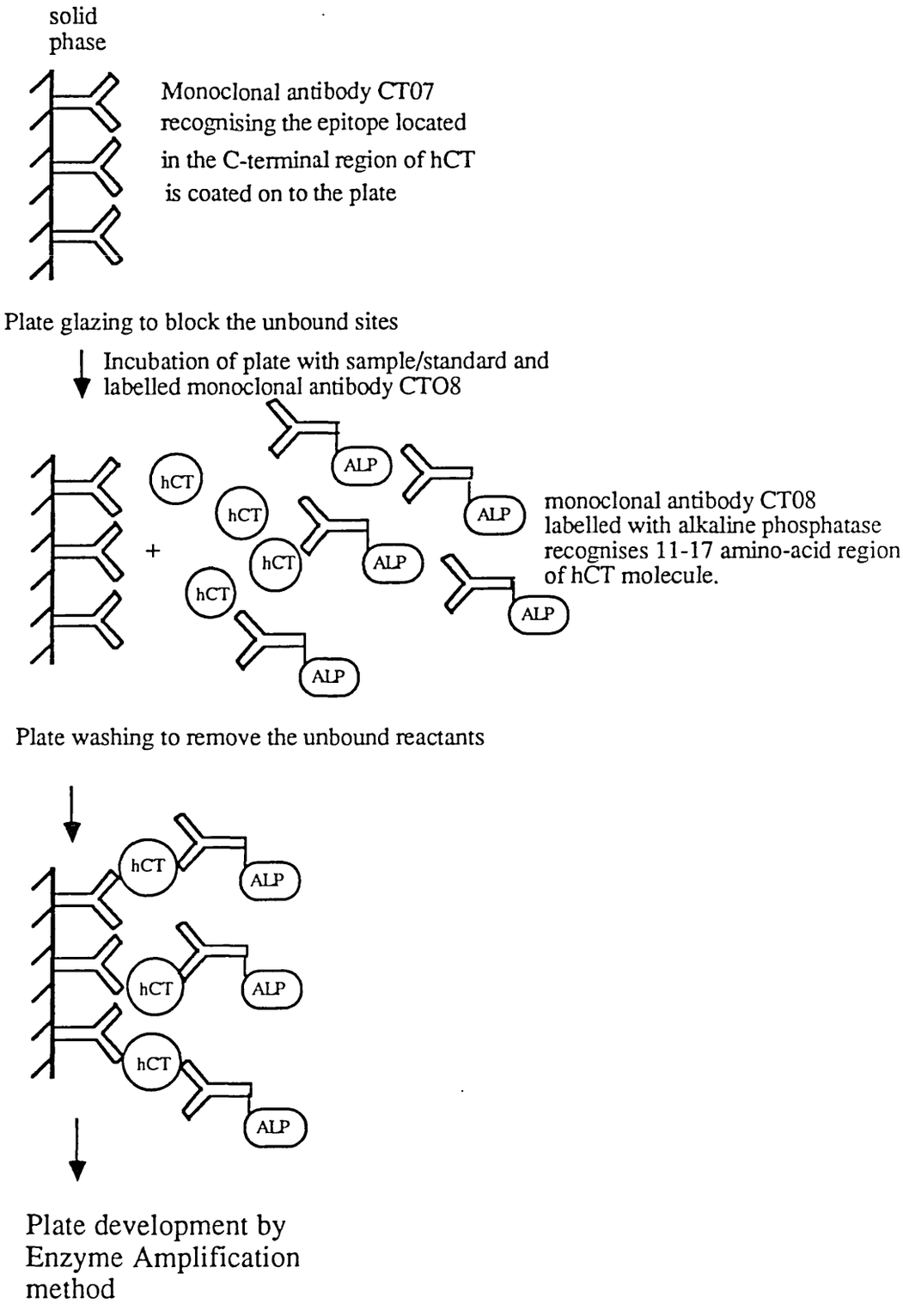


Figure 25: Assay format for the monoclonal antibody-based EIA for hCT

have an amidated proline residue. Enzyme amplification was also incorporated into the assay method to achieve greater speed and sensitivity.

The new assay was developed and applied to the measurement of i-hCT levels in normal volunteers, patients with MTC and post-menopausal women.

There have been claims that hCT levels are higher in African blacks than in whites (Stevenson, Myers and Ajdukiewicz, 1984). However, the number of subjects included in the study was small (6). Therefore, a study was planned to measure hCT levels in black people of West Indian origin in comparison with age-matched white controls.The reason for this study was that osteoporotic fractures are relatively rare in black populations of Caribbean or African origin. Many possible reasons have been advanced, and it was important to determine whether any difference in the hCT levels is a factor.

Conflicting reports on the hCT levels in osteoporotic patients have been published (Samaan, Anderson and Adam-Mayne, 1975; Deftos *et al.*, 1980). The new assay was used to measure i-hCT in normal post-menopausal women to find out whether they have low or normal i-hCT.

The existence of multiple hormonal forms for hCT has been well documented (see page 65). A gel-filtration study was carried out to test the specificity of the new assay for the particular immunoreactive form of the peptide. Immunoreactive hCGRP and katacalcin were also measured (by RIA) to obtain the complete profile of the calcitonin gene peptides in patients with MTC.

Part 1

4.2. The monoclonal antibody-based enzyme immunometric assay

Materials and methods

4.2.1. Buffers

The assay buffers used in this part of the study were the same as those used earlier.

4.2.1.1. Coating buffer (see page 79).

4.2.1.2. Glazing buffer (see page 80).

4.2.1.3. Assay buffer (see Page 80).

4.2.1.4. Chromatography buffer

Ammonium acetate 0.02M pH 6.4 buffer containing sodium azide (0.02% w/v).

4.2.2. Enzyme amplification kits (Ampaks) (see page 79)

4.2.3. Plasma purified fraction (PPF) (see page 80)

4.2.4. Antibodies

Monoclonal antibodies (designated CT07 and CT08) were raised in the Institute Gustave-Roussy, and their characteristics reported earlier (Motte *et al.*, 1986). Antibody (CT08) was labelled with alkaline phosphatase as before by the method of Ishikawa *et al.*, (1983). The conjugation was performed by Dr. James Q. Fuller of Novo Biolabs, Cambridge.

The polyclonal antiserum (designated OC31) used in the conventional RIA

(see chapter 2)was also used in combination with monoclonal antibodies. Table 12 shows the summary of antibody characteristics.

4.2.5. Radiolabelled hCT (see page 79).

4.2.6. Samples

Normal volunteers

Fifty-three normal, healthy volunteers were recruited. They were divided into two main groups; males (age range 17-70, n=22) and females (age range 17-52, n=31). Ethical Committee approval was obtained in addition to the informed consent of the volunteers. Peripheral blood was obtained in cold heparinised tubes after an overnight fast. Samples were centrifuged at 4°C for ten minutes at 900g and the plasma was stored at -20°C until the time of assay. Each sample was analysed by the two-site EIA and the conventional RIA (using the long incubation protocol of 7 days because of the low hCT levels).

MTC patients

Peripheral venous plasma samples were obtained from 20 patients with MTC in the same way as for normals. Each sample was assayed by both the monoclonal antibody-based EIA and the overnight RIA.

Post-menopausal women

Normal, caucasian, post-menopausal women (age range 48-70, n=25) were recruited. Samples were treated in a similar fashion to those from the normal volunteers.

Black (negroid) volunteers

Twenty-five normal, black people who had lived in this country for a number of years were recruited. Samples were treated as above.

4.3. Immunoassay development

4.3.1. Optimisation of assay

4.3.1.1.Selection of the best pair of antibodies

Although the monoclonal antibodies were available, it was necessary to investigate the possible use of the existing polyclonal (OC31) antiserum in combination with the monoclonal antibodies. The characteristics of the antibodies used are summarised in table 12. A chequer board diagram (see table 13) indicates the combination of antibodies investigated. The procedure involved coating three microtitre plates in duplicate with the three antibodies (CT07, CT08 and OC31) at an initial dilution of 1: 1000 (100 µl/well). Synthetic hCT was serially diluted in PPF and duplicate plasma or standards (100µl) were dispensed into the microtitre wells. The enzyme-labelled antibodies (CT07, CT08 and OC31; 100µl/ well, 1:1000 initial dilution) were then added to the appropiate plates without any delay. The plates were incubated at room temperature overnight to establish the best antibody combination for a sensitive assay. At the end of the incubation, the reactants were shaken out and the plates were developed as before using the enzyme amplification method.

CT07/CT08 combination produced the most satisfactory result (see table 13).

4.3.1.2. Selection of enzyme-labelled antibody concentration

A similar experiment was set up with to investigate the optimum amount of enzyme-labelled antibody (CT08) required to give a sensitive assay. A plate coated with CT07 (100µl/well; 1:1000 initial dilution) was glazed as before, and serially diluted hCT standards (100µl/well; 0-10 ng/ml) in PPF were added in duplicate. Varying dilutions of the enzyme-labelled antibody in the assay buffer were prepared and then added to the wells and the plate which was incubated overnight at room temperature. At the end of the reaction, the plate was washed and developed as before. The optimum amount of enzyme-labelled antibody for

use in the assay was selected as 1:1000 initial dilution.

4.3.1.3.Selection of assay incubation time

An experiment was set up as follows to investigate the optimum assay time required. The plates were coated with CT07, glazed as before and a range of diluted standards (100 µl ; 0-10 ng/ml) were then added as before. The enzyme-labelled antibody (100µl; 1: 1000 initial dilution) in assay buffer was then added to all the plates and incubated at varying lengths of time (0.5 hr, 1.0 hr, 2.0 hrs, 4.0 hrs, 16 hrs, 24 hrs and 48 hrs). The plates were then washed and developed as before to determine which incubation time gave the most sensitive assay.

The overnight protocol was adopted since it provided the most sensitive assay for the measurement of low levels of calcitonin (see figure 27).

4.3.1.4.The effect of different media on the calibration graph

An experiment was set up using the best conditions determined from the previous experiments to see the effect of using Tris buffer, phosphate buffer and PPF on the hCT calibration graph. The experiment was conducted in an identical manner as above except that three sets of standards serially diluted in three different media were placed in the wells of the previously coated and glazed plate. The enzyme-conjugated antibody was added and incubated overnight. The plate was then washed and developed as before and calibration graphs were constructed to compare the results (see figure 27).

4.3.2. Calibration graph for hCT

The final conditions for the hCT assay were as follows. The antibody used for the coating of the plates was CT07 which recognised the C-terminal amidated hCT and was used at an initial dilution of 1:1000 (50µg/ml). Synthetic hCT standard was diluted serially in PPF and duplicate plasma or standards (100µl) were dispensed into microtitre wells. The enzyme-labelled antibody (CT08) was added (100µl/well) at an initial dilution of 1:1000 and the plates incubated overnight.The plates were then washed and developed as before. The sensitivity

of this assay was in the region of 1-2 pg/ml.

4.3.3. RIA

The RIA employed was the same as that reported in chapter 2 with a slight modification in the assay incubation times. When measuring hCT in normal volunteers and post-menopausal women, an incubation time of 7 days (4 days incubation of samples with antibody followed by the addition of labelled hCT and a further incubation of 3 days) was employed. In plasma samples and gel-chromatography fractions containing high levels of hCT, an overnight incubation protocol was employed.

4.3.4. Inter and Intra-assay variations

Two quality control pools were prepared containing 16 pmol/l and 32 pmol/l of synthetic hCT. Each pool was assayed 20 times in one assay and then repeatedly in 10 separate assays.

4.3.5. Recovery

Synthetic hCT (46.8 pmol/l) was added to 17 normal plasmas which were analysed before and after the addition of the peptide. The mean percentage recovery was determined.

4.3.6. Sensitivity and specificity

The sensitivity of the assay was determined as before (chapter 2) to give the lowest amount of i-hCT that could be determined in the assay with 95% confidence.

The peptides that were tested for cross-reactions at dose levels of up to 100 fold greater than the synthetic hCT were:- salmon calcitonin, eel calcitonin, porcine calcitonin, katacalcin, rat CGRP, human CGRP, ACTH, bombesin, FSH, LH, LHRH, TSH, GH, insulin, angiotensin ii, angiotensin iv, pre-opiomelanocortin (POMC), substance P, vasopressin and glucagon. There was no cross-reaction of any peptide with the monoclonal antibody-based

immunoassay.

4.3.7. Fast assay for hCT

An assay for hCT was attempted in which the total assay time (including development of the plate) was reduced to just 20 minutes. The samples loaded on the plate were undiluted plasma samples from MTC patients. The concentration of the labelled monoclonal antibody was increased to 1:100 (initial dilution). The incubation times for the enzyme amplification method were also reduced (5 minutes for substrate incubation and addition of amplifier solution). The colour development was rapid (within 5 minutes). The results of this experiment are shown in figure 33.

4.4. Results of the monoclonal antibody-based assay

A pair of monoclonal antibodies obtained from the Institut Gustave-Roussy, France were used to develop an EIA for hCT. The characteristics of these antibodies have been described earlier (Motte *et al.*, 1987). This information is summarised in table 12. A polyclonal antibody designated OC31 used in chapter 2 to develop a one-site AEIA was also tested for its performance in a two-site assay.

A "chequer-board" experiment as shown in table 13 was performed using all three antibodies. The results obtained are shown in figure 26. It was found that when labelled OC31 was used in combination with CT07 as the solid-phase antibody (as in figure 26c), the resulting standard curve was flat. The reason for this observation was that CT07 and OC31 appear to recognise the same epitopes (C-terminal, 26-32 region). Therefore, there in no formation of a "sandwich" and hence a flat standard curve. When OC31 was used as the solid-phase antibody in combination with CT07 as the labelled second antibody, then a reasonable dose-response curve was obtained, as shown in figure 26f.

When OC31 was used in any combination with CT08 (as in 26b and 26d), a good dose-response curve was obtained. This was probably because the two antibodies recognised different epitopes.

However the best response was obtained by using a combination of the two-monoclonal antibodies (as in 26a and 26e), particularly when the solid-phase antibody was CT07 (as in 26a). The reason for this was not known but it can be speculated that the sensitivity of a two-site assay is determined by the affinity of the labelled antibody. In this case the affinity constant of CT08 was greater than that of the solid-phase bound CT07 which resulted in the most sensitive assay.

From the above observations it was concluded that the epitope recognised by the polyclonal antibody OC31 was most probably located in the C-terminal region of the hCT molecule. Having selected the best combination of the pair of

Antibody	Type	Isotype	Affinity constant (l/mol)	Epitope recognised
CT07	monoclonal	IgG2a	0.9×10^{10}	26-32 region
CT08	monoclonal	IgG1	3×10^{10}	11-17 region
OC31	polyclonal	IgG	7.4×10^{9}	mainly C-terminal

Table 12: Characteristics of the anti-hCT antibodies used in the development of a two-site assay for hCT.

Solid phase antibody \ Enzyme-labelled antibody	CT08*	CT07*	OC31*
CT08	Not performed	+	++
CT07	+++	Not performed	+
OC31	++	+	Not performed

Table 13: Chequer board diagram to show the effect of using different antibody combinations. (+) Indicates the sensitivity of the resulting calibration graphs.

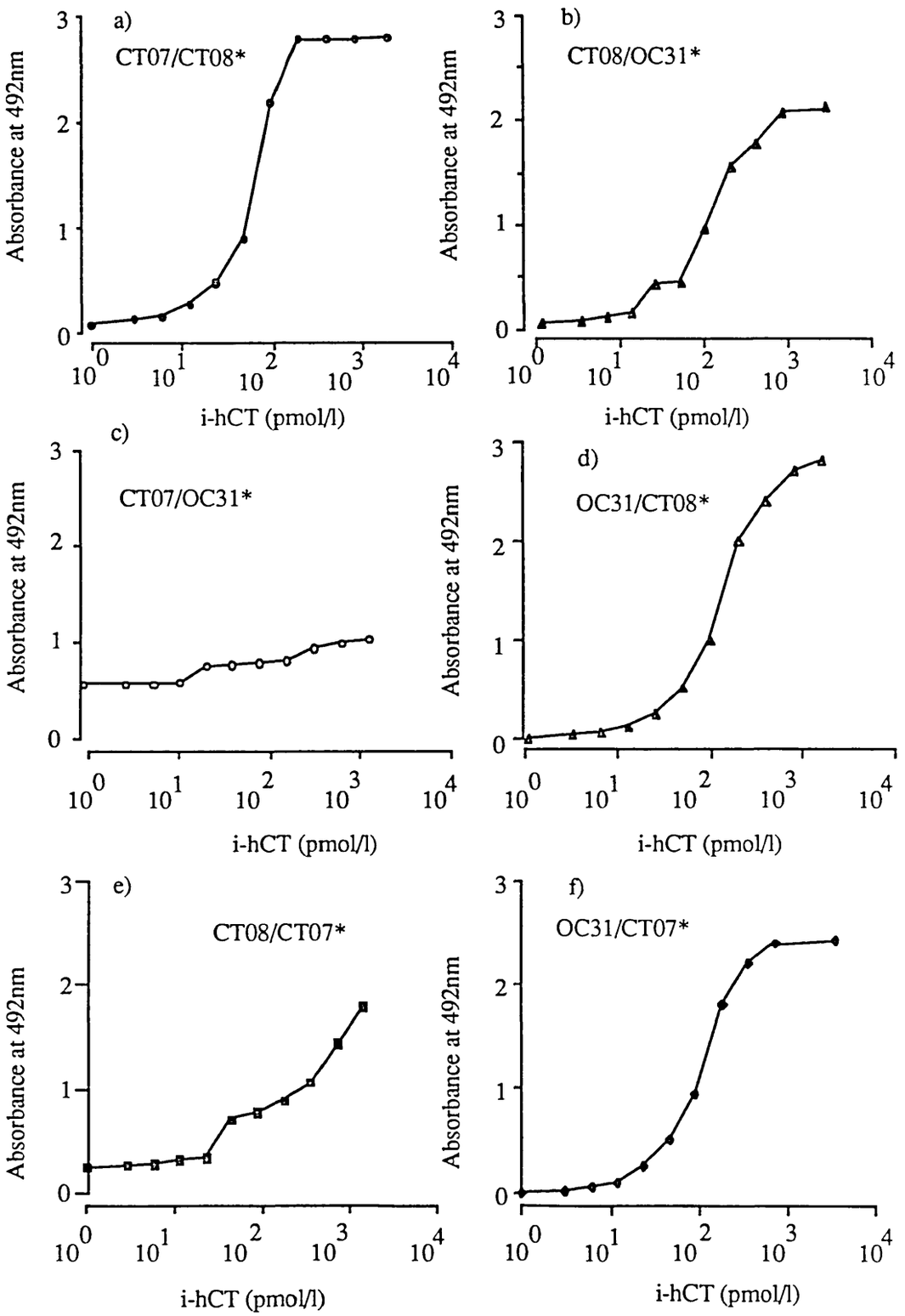


Figure 26: The effect of varying the antibody combinations

antibodies, the assay was optimised in terms of which conjugate concentration, incubation time and assay medium to use in the final calibration graph.

Figure 27a shows the effect of using different labelled antibody fractions after a purification procedure. The least sensitive dose-response was obtained with fraction 6 whereas fractions 2, 3 and 5 gave similar responses. The best result was obtained by using fraction 4 of the labelled antibody conjugate.

In figure 27b, the result of varying the reaction incubation time is shown. The most sensitive dose-response curve was obtained using an overnight incubation protocol. However, the incubation time of the assay can be shortened (with a loss of sensitivity from 2 pmol/l to 20 pmol/l) in order to measure i-hCT in patients with MTC where the levels are grossly elevated, or during column purifications where it is necessary to know the result of an experiment quickly in order to proceed to the next stage. Further work carried out to develop fast assays is discussed in chapter 5.

The effect of varying the assay buffer is shown in figure 27c. Tris and phosphate buffer gave virtually superimposable dose-response curves. Since the assay was mainly developed in order to measure the i-hCT in plasma, PPF was used in the assay since plasma samples were being quantitated.

A typical developed plate is shown in plate 5 and an EIA dose-response graph for the measurement of i-hCT is shown in figure 28. Also shown on the same figure are parallel dilutions at 1:100 and 1:500 of plasma obtained from 5 patients with MTC. The dose-response curve was found to be linear in the range of concentrations up to 150 pmol/l. The assay sensitivity was found to be 1.7 pmol/l whereas that of the corresponding overnight RIA was 10 pmol/l.

The sensitivity of 100 assays over a period of 18 months did not alter significantly and remained unaffected by the age of the labelled antibody.

The inter and intra-assay variation for the two pools were 10.8 and 11.3 %

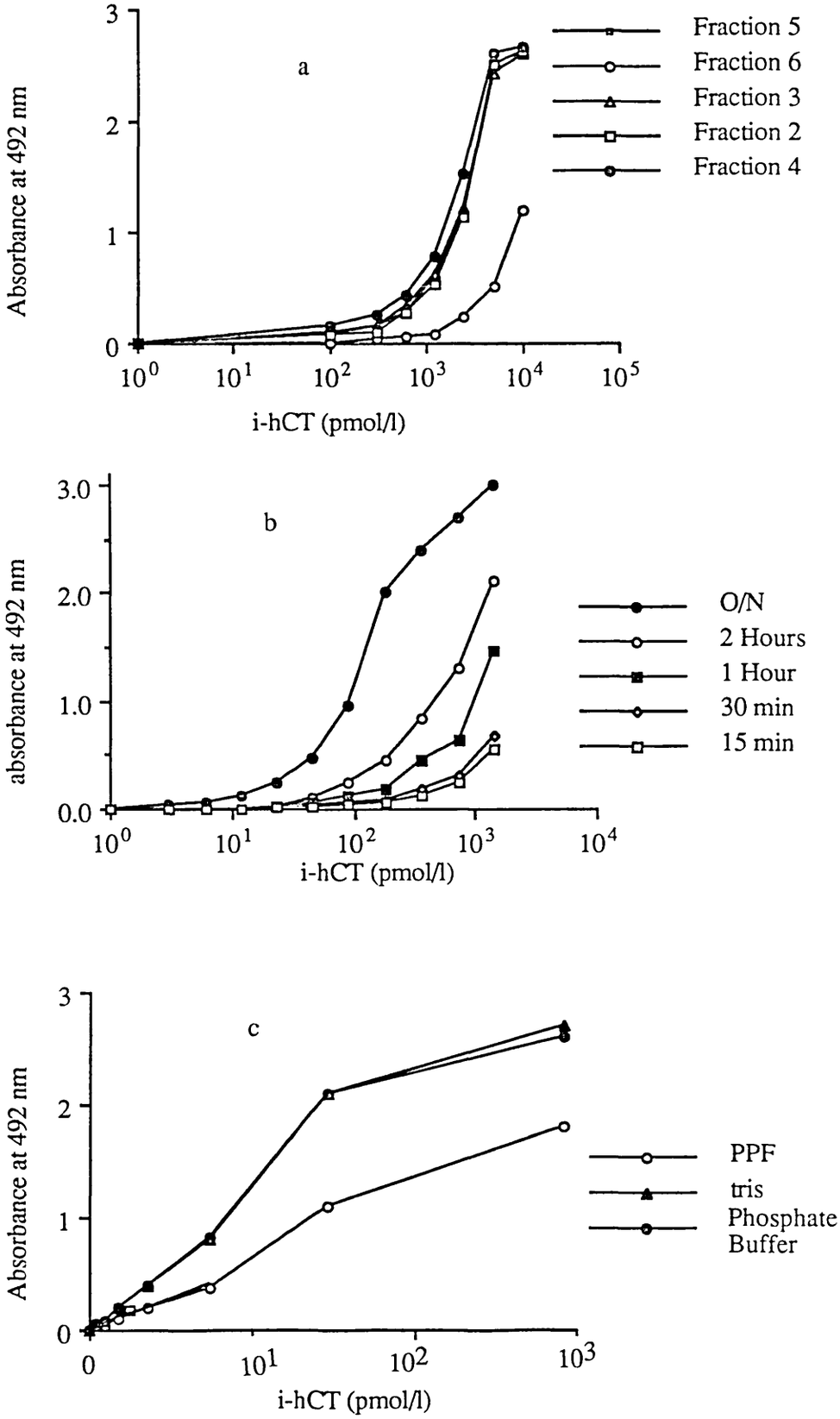
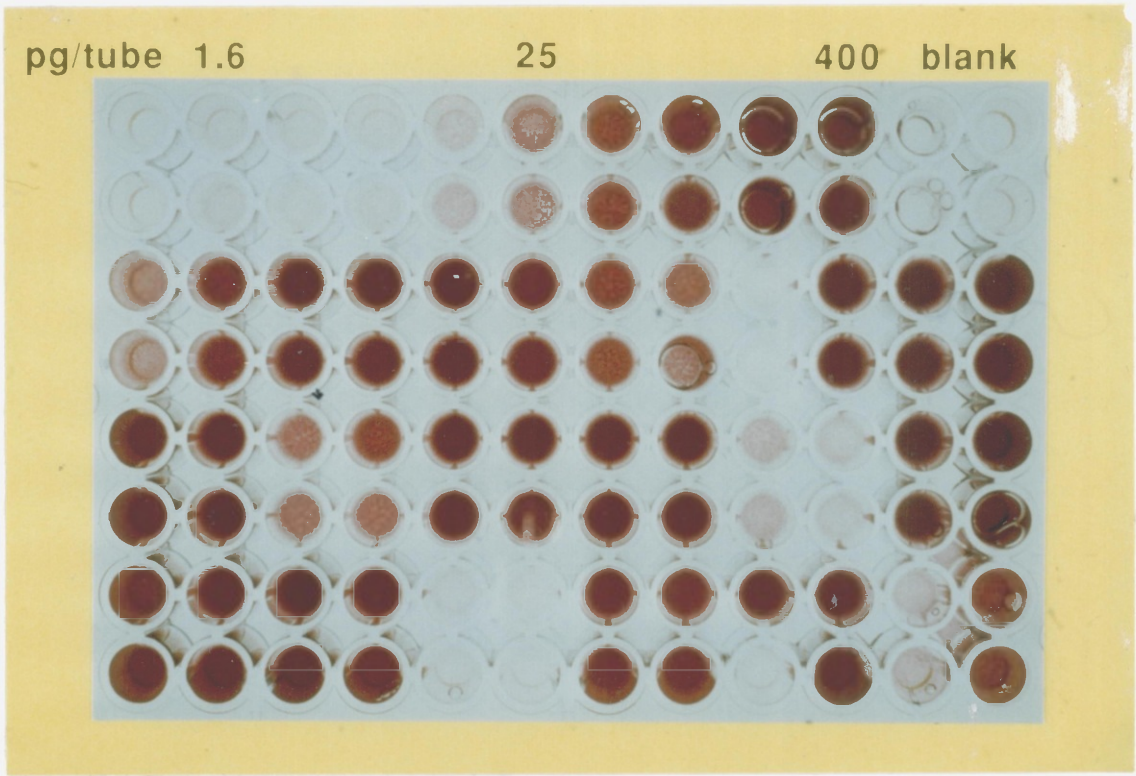


Figure 27: The effect of varying conjugate, time and assay buffers on the hCT calibration graph

Plate 5. An example of the monoclonal antibody-based two-site EIA developed using enzyme amplification.



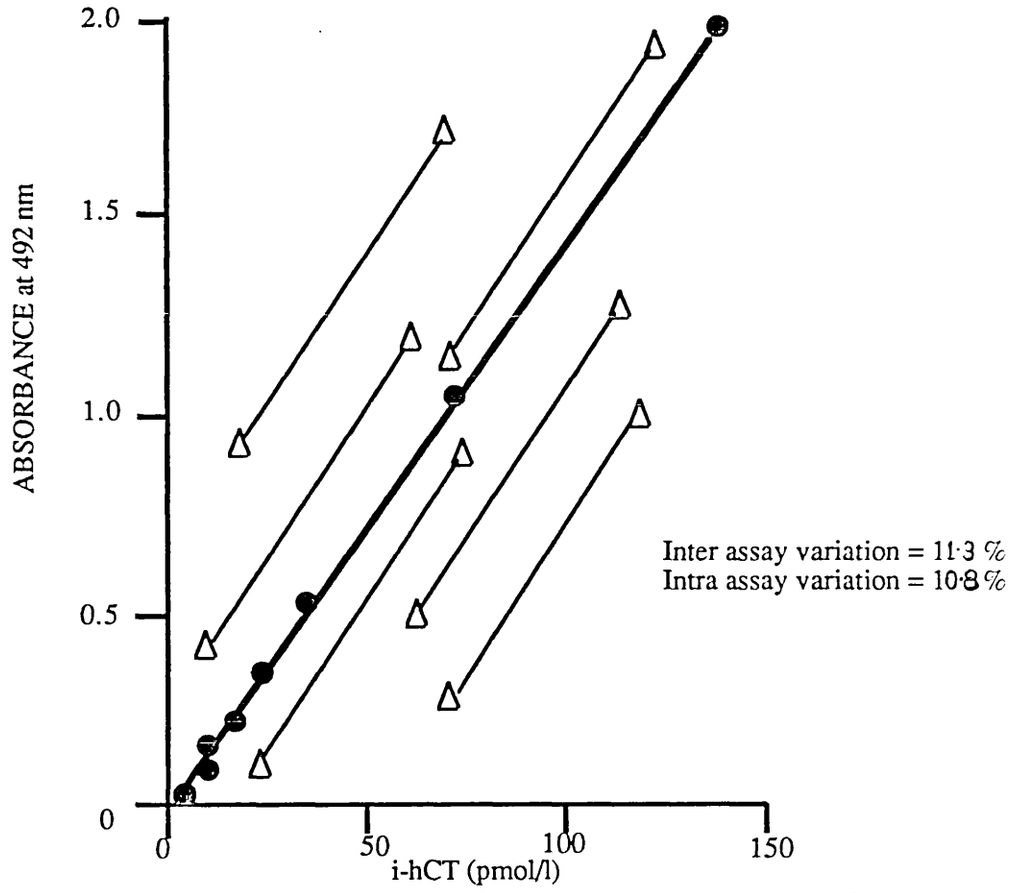


Figure 28: Calibration graph for the measurement of i-hCT in plasma (●). Plasma from patients with MTC (Δ) shows parallelism to hCT standard

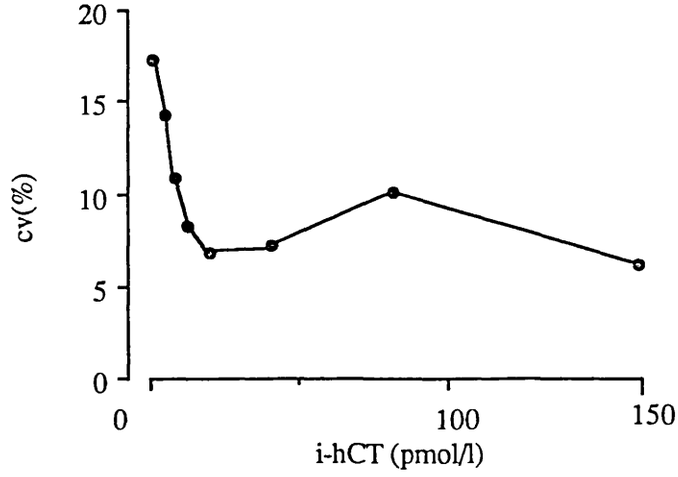


Figure 29: Dose-error relationship for i-hCT assay constructed by plotting the % CV against each dose

respectively. A dose-error relationship, shown in figure 29, was constructed using estimates of variance from replicates at each dose level from 10 standard calibration graphs to give a working range between 2 and 150 pmol/l.

The mean recovery of the added synthetic hCT (46.8 pmol/l) to the plasma of normal volunteers was 96% (44.9 ± 4 pmol/l).

In terms of specificity, no binding of a number of peptides, even when used at a concentration 100 times greater than that of the highest standard,was detected in the EIA.

Circulating i-hCT levels

In figure 30, the levels of hCT in MTC patients measured by EIA and the overnight RIA are shown. Both assays detect highly elevated levels but the mean levels measured by RIA were significantly higher ($p < 0.001$, students T-test) than those measured by the EIA (see table 14). In normal volunteers, the median levels of hCT measured by EIA in men and women were found to be 6.4 and 2.6 pmol/l respectively whereas those measured by RIA were 8.8 and 6.1 pmol/l. In women, there was a significant difference ($p = 0.02$, Wilcoxon's matched pairs test) between the levels measured by EIA and RIA (see figure 31).

The sex difference in circulating i-hCT levels measured by the two assays is shown in figure 31 and was found to be lower ($p < 0.05$, Mann Whithey U-test) in women (median 2.6 pmol/l by EIA and 6.1 pmol/l by RIA).

A summary of the plasma levels of i-hCT in negroid and caucasian subjects is shown in table 15. There was no significant difference in the median levels between these groups. However, there was a significant difference ($p < 0.05$, Wilcoxon's matched pairs test) between the EIA and the RIA.

Immunoreactive hCT levels in post-menopausal women is summarised in table 14 and shows low levels of i-hCT after the menopause.

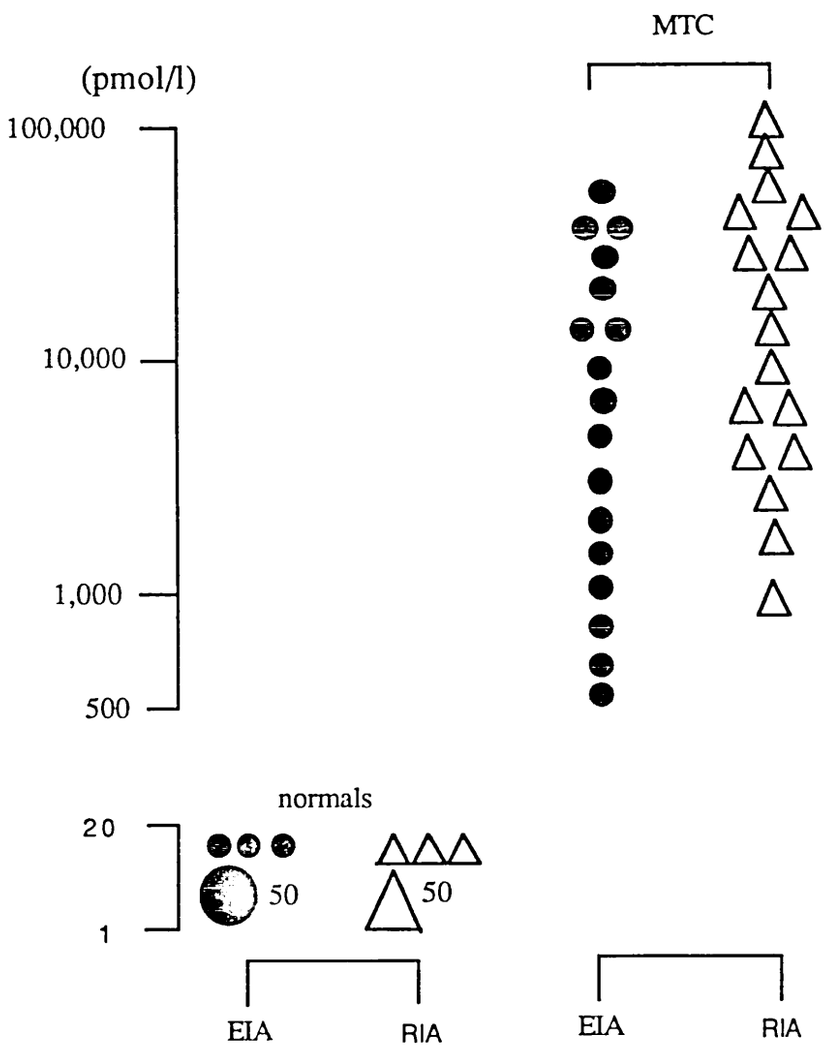


Figure 30: Concentrations of i-hCT in plasma as measured by RIA and EIA in normal men and women (n=53) and 17 patients with MTC. Levels of i-hCT are highly elevated in MTC

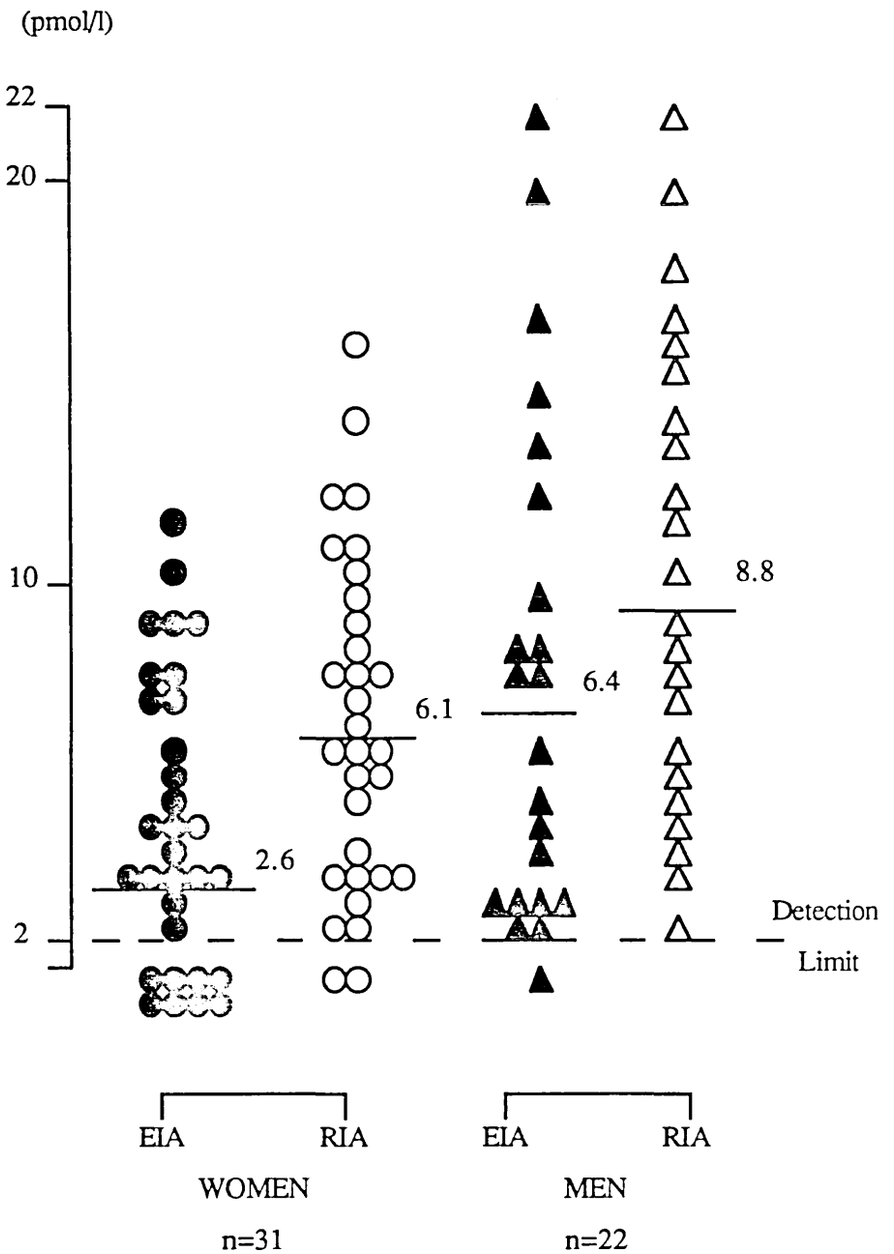


Figure 31: **Levels of i-hCT in normal men and women as measured by RIA and the two-site EIA. The horizontal bar represents the median value in each group**

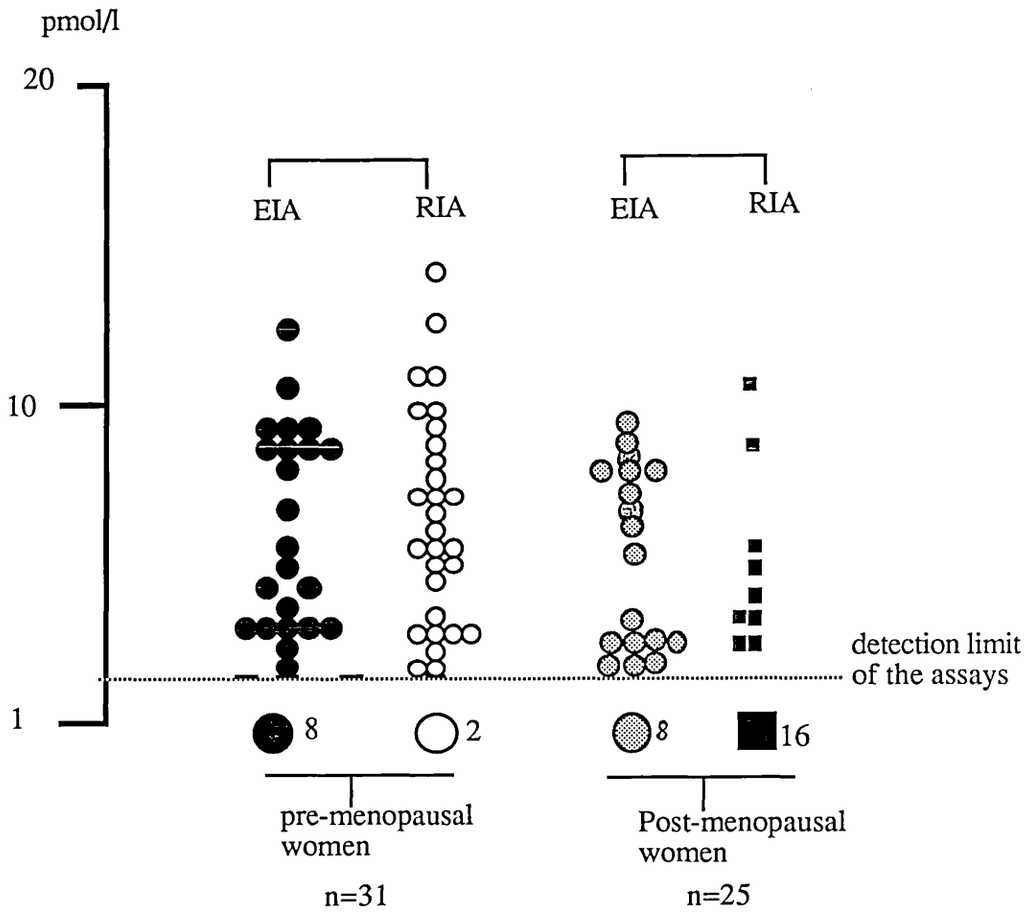


Figure 32: Levels of i-hCT in pre- and post menopausal women as measured by RIA and the two-site EIA.

RIA vs EIA

	RIA	EIA		
	median value (pmol/l)	median value (pmol/l)	n	p-value
Normals	6.44	3.8	53	0.005
Males	8.8	6.4	22	0.05
Female	6.1	2.6	31	0.02
MTC	34,968	10,140	17	0.001
Post-menopausal women	16/25 undetectable	8/25 undetectable	25	

Table 14: Summary of the results obtained by EIA and RIA in normals, MTC patients and post-menopausal women together with the statistical data

	RIA (pmol/l)	EIA (pmo/l)	n	p value
White	6.2	3.2	42	0.05
Black	4.8	2.6	25	0.05
p value	NS	NS		

Table 15: Comparison of the i-hCT levels as measured by RIA and EIA in normal black and white people

Fast assay

The application of an enzyme amplification system in this study has indeed contributed significantly in not only increasing the sensitivity but also the speed at which these assays can be carried out. Figure 33, shows a calibration graph obtained from an enzyme-immunometric assay for hCT using monoclonal antibodies carried out in 20 minutes. The sensitivity of such an assay is reduced to 50 pmol/l but in a clinical sense, the speed of such an assay should prove very useful where critical decisions have to be made on the basis of circulating peptide levels, for example, in the pre- and post-operative treatment of MTC.

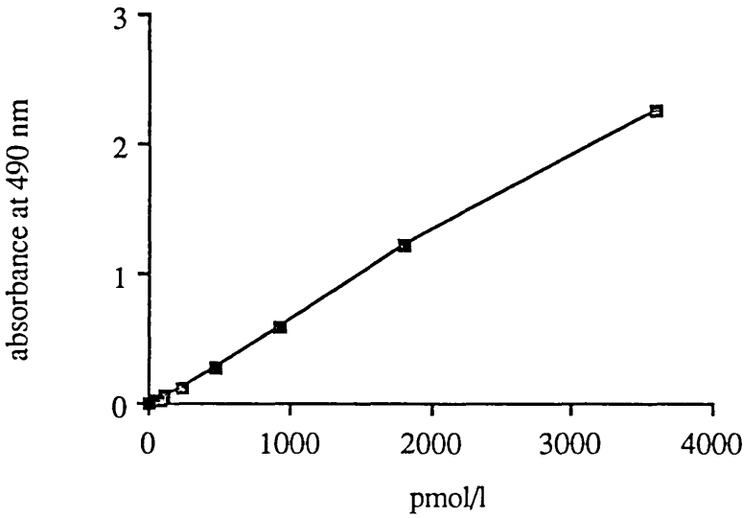


Figure 33: Fast (20 minutes) EIA for i-hCT

Part 2

4.5. Gel-permeation chromatography

4.5.1. Aim of the study

During the course of the present study, it was observed that the i-hCT levels detected by RIA were higher than those detected by the monoclonal antibody-based EIA (see table 14). To investigate this discrepancy, gel-filtration studies were undertaken followed by measurement of i-hCT in all the eluted fractions by both RIA and EIA to determine the specificity of each assay for the particular molecular size of the peptide. Immunoreactive hCGRP and katacalcin were also measured by RIA. The scheme for the gel-filtration study is shown in figure 34.

4.5.2. Pilot study

A preliminary study was conducted to optimise the experimental conditions and calibrate the chromatography columns. Plasma (12 ml) from one MTC patient was obtained, lyophilised and reconstituted in a smaller volume (6 ml) of the chromatography buffer (0.02 M ammonium acetate buffer, pH 6.4). Aliquots of this (2ml) were chromatographed as follows.

Sephadex G-50 superfine (10 g, Pharmacia, Uppsala, Sweden) was allowed to swell in the chromatography buffer overnight at 4°C and packed into a column (1.5 cm internal diameter x 85 cm). The packed column was equilibrated at 4°C in the same buffer. Reconstituted sample (2 ml) was loaded on to the column. Fractions (3ml) were eluted at a flow rate of 1 ml /10 minutes in the same buffer. Corresponding fractions were pooled from three chromatographs, lyophilised, reconstituted into phosphate buffer (1ml) and assayed for the presence of albumin (void volume), hCT, katacalcin and hCGRP.

This represents stage 1 in the scheme shown in figure 34.

4.5.3. Measurement of albumin (Lowry's protein estimation)

Lowry's method (Lowry, Rosebrough, Farr and Randall, 1951) of protein estimation was used to quantitate the amount of protein in the column fractions. The method was scaled down to enable the use of microtitre plates and the

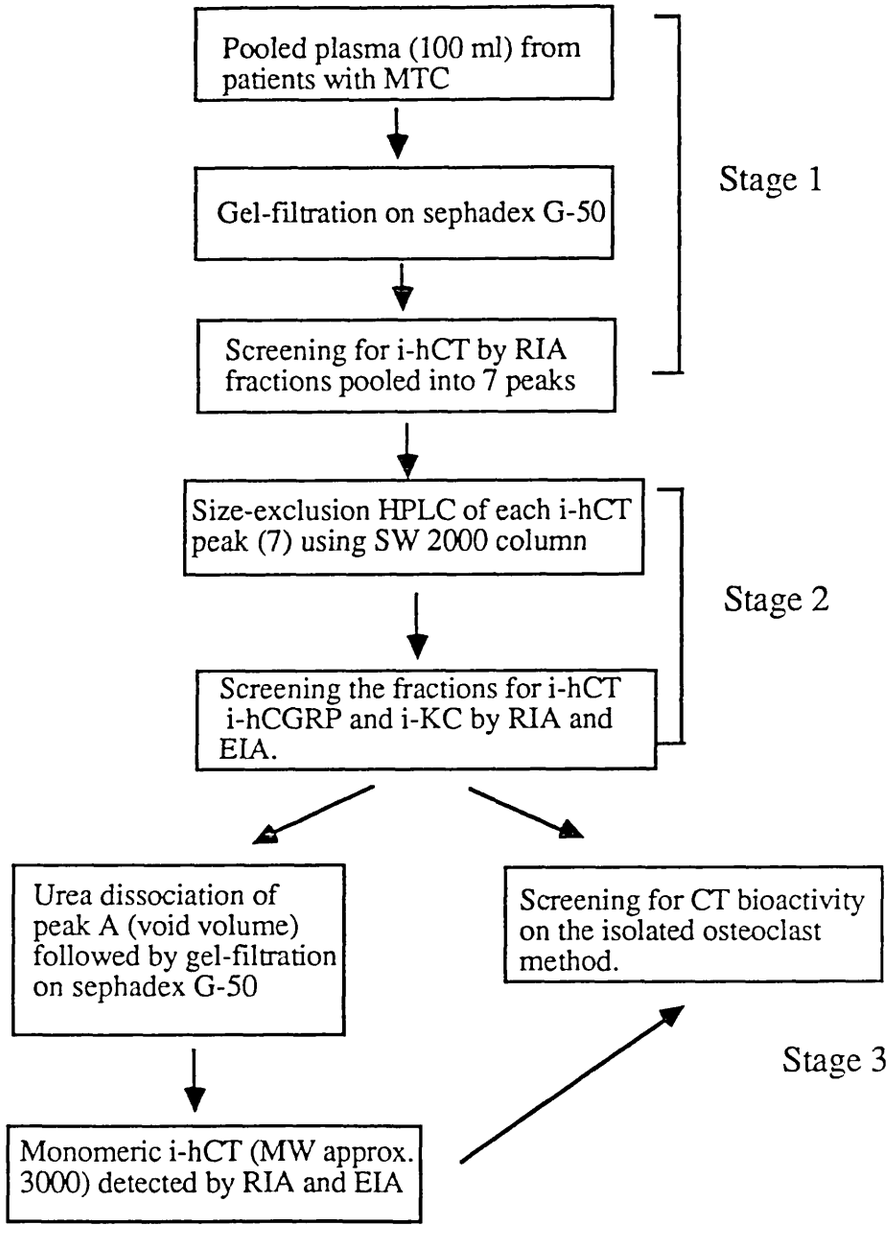


Figure 34: Schematic representation of the gel-filtration studies

volumes of the reagent were adjusted accordingly.

Reagent A	2% (w/v) sodium carbonate solution in 0.1M sodium hydroxide solution
Reagent B	0.5% (w/v) copper sulphate solution in 1% (w/v) sodium potassium tartrate solution.
Reagent C	50 ml of reagent A + 1ml reagent B
Reagent D	Folin & Chiocalteu's phenol reagent (1:2 dilution)
	Human serum albumin 10mg/ml standard preparation

Human serum albumin standard was serially diluted in phosphate buffer to obtain the following range of concentrations 10, 5, 2.5, 1.25, 0.64, 0.32, 0.16 and 0 mg/ml.

Standards and samples (10 µl/well) were dispensed in duplicate into the wells of a micro-titre plate. Reagent C (100µl/well) was added to all the wells and the plate incubated for ten minutes at room temperature. Reagent D (10µl/well) was added to all the wells and the plate was further incubated for 40 minutes. The absorbance of each well was determined at 690nm using a Titertek Multiscan ELISA plate reader. The protein concentration was determined in all the fractions.

4.5.4. Immunoassays

4.5.4.1. Calcitonin measurement

Immunoreactive hCT was measured by EIA and RIA as described earlier. The column fractions were lyophilised to remove the ammonium acetate buffer, reconstituted in the assay buffer (1 ml) and quantified by both the EIA and the RIA.

4.5.4.2. Katacalcin

A RIA for katacalcin was reported by Hillyard *et al.*, (1983). A tyrosine-containing analogue of KC had been synthesised for the purpose of radiolabelling and for the raising of antisera.

Katacalcin antiserum was raised by immunising rabbits with ovalbumin-katacalcin conjugate. The antiserum was tested for cross-reactions with various peptides as used before for hCT (chapter 2) and was found to be specific for katacalcin.

Radio-iodination of tyrosinated katacalcin was carried out as before using the chloramine-T method (Greenwood, Hunter and Glover, 1963) and the labelled peptide was purified on reverse phase-HPLC using an elution gradient of 10-37% acetonitrile (ACN) with the peak eluting at 24% ACN. Protein (4.5% w/v albumin) was added to prevent the labelled peptide sticking to the walls of the tube. The tracer was stored at -20°C.

The immunoassay was performed as a disequilibrium assay. The samples (reconstituted column fractions, 100µl/tube) were incubated with antibody diluted at 1/10,000 (initial dilution) for 2 days. The tracer (containing 10,000 counts/tube) was then added and further incubation carried out for 24 hours. At the end of the reaction, sheep anti-rabbit antibody bound to cellulose was added and the separation carried out as described earlier. The bound fractions were measured for radioactivity. The assay sensitivity was 0.02 nmol/l, the inter and intra-assay variations were 7 and 5 % respectively.

4.5.4.3. Human CGRP RIA

The CGRP assay employed here is the same as that reported earlier (Girgis *et al.*, 1985). The radiolabelled tracer was purchased from Amersham International, Amersham, Bucks. CGRP antiserum designated CC21 was raised in rabbits and was specific for CGRP alone (see chapter 2).

The assay was a disequilibrium assay. The antibody (1/40,000 initial dilution) was first incubated with the samples and standards for 3 days. The tracer was then added and a further incubation was carried out for 2 days. The separation method was the same as that employed for katacalcin and calcitonin RIA.The sensitivity of the assay was found to be 0.013 nmol/l, and the inter and intra-assay variation were found to be 9% and 6% respectively.

4.5.4.4. Large scale gel-filtration study

Plasma (100 ml) from various MTC patients was collected over several months, pooled, centrifuged and lyophilised after the addition of aprotinin to inhibit endogenous proteinases. It was then reconstituted (20 ml) into chromatography buffer (ammonium sulphate, 0.02M pH 6.4) and divided into five aliquots and chromatographed under the same conditions as described earlier. The corresponding fractions were pooled, lyophilised, and scanned for the presence of i-hCT by RIA. The fractions were then pooled into 6 main peaks A, B, C, D, E and F.

Peaks B to F were further purified using size-exclusion, calibrated HPLC gel-chromatography on a SW2000 column as shown in figure 34. The fractions were scanned for the presence of i-hCT by RIA and EIA and katacalcin. A UV absorbance trace was also obtained for all the fractions.

Peak A (the void volume peak) was further divided into three aliquots. Aliquot one was treated with 8M urea to dissociate the protein bound i-hCT. The reaction mixture was then subjected to gel-filtration on a sephadex G-50 column. The fractions were eluted in the buffer (containing 8M urea), desalted on a sephadex column (PD 10), lyophilised, reconstituted in RIA buffer and the i-hCT present in the fractions was measured by RIA and EIA. The second aliquot was used as a control and chromatographed without treatment with urea. The eluates were measured for i-hCT as before. Bioassay was carried out on all the fractions well as the controls. The biological activity of the fractions corresponding to the monomeric i-hCT obtained from the urea treated peak A were also assessed in this way.

4.5.5. Bioassay of i-hCT fractions

The bioassay described below was performed by Dr. Zaidi and Dr. Datta (Dept. Chem Path, RPMS). A recently developed bioassay based on calcitonin induced inhibition of motility of freshly disaggregated rat osteoclasts isolated from neonatal long bones was employed (Chambers and Moore, 1983; Zaidi, Chambers, Bevis, Beacham, Gaines Das and MacIntyre, 1988). This assay has been used to detect biological activity in the eluted fractions containing i-hCT in plasma of MTC patients following gel-filtration.

A neonatal rat (less than five days old) was sacrificed. The long bones were detached and cut into small segments in a petri dish in the presence of culture medium containing 20% FCS. The bone slices were allowed to incubate for a short time for the cells to become released into the medium. The debris was removed and the cells were then washed by placing fresh medium into the dish.

The cells were then incubated at 37°C for 30 minutes and observed under the microscope, counted and scanned for motility.

A standard preparation of hCT (10 ng/ml) was serially diluted for the preparation of a calibration curve. The column fractions (peaks A to G) corresponding to the presence of i-hCT were prepared. A microtitre plate containing 24 wells was taken and the isolated osteoclasts were placed in the wells of this plate so that each well had at least 10 cells.

The standards and samples were added to the wells and incubated for one hour. The immotile cells were counted in each well and expressed as a percentage of total cells. A calibration graph was obtained (see figure 36). The biological activity in the peaks containing i-hCT was assessed from this calibration graph.

4.6. Results

Gel-filtration

A calibration graph of the size-exclusion chromatography is shown in figure 36. The result of gel-filtration of MTC plasma from one patient (pilot study) is shown in figure 35. The recovery of the i-hCT from the gel-filtration column was 74% as measured by the RIA. Multiple immunochemical peaks were detected for all three peptides. CGRP circulated in a much lower concentration than hCT and KC. The majority of the i-hCT eluted earlier than the synthetic monomeric form, indicating higher molecular forms. Levels of i-hCT in the fractions measured by EIA and RIA were similar except in the void volume region where the levels measured by RIA were higher than those by the EIA. The fragment peak indicated in the salt volume region of the column (Vt) was not detected by EIA.

The main study was conducted in an identical manner as the pilot study. After the initial gel-filtration (stage 1) the corresponding fractions from 5 identical and separate chromatograms were pooled and divided into 5 major peaks and 2 minor peaks. All the peaks except peak A were further purified on size-exclusion HPLC. The results obtained are shown in figures 37-43. Peak E corresponded to the monomeric hCT where as peaks B to D represented higher MW i-hCT (probably dimers and tetramers). Peak A represented the protein-bound i-hCT.

In figures 37 and 38, all the immunoreactive peaks for hCT and KC as measured by RIA are shown. Figure 39 shows individual peaks of i-hCT as measured by RIA and EIA. The dotted line indicates the elution position of the monomeric hCT. Peak E is the monomeric i-hCT in the MTC plasma. In figures 40-43, individual peaks of i-hCT (and KC) together with the UV absorbance trace are shown to confirm the specificity of the RIA and EIA. Figure 44 shows the elution profile of hCTfrom peak A before and after urea treatment and figure 45 summarises the profile of all the peaks as measured by RIA and EIA in MTC plasma.

The results of the bioassay are shown in figure 46 and discussed later.

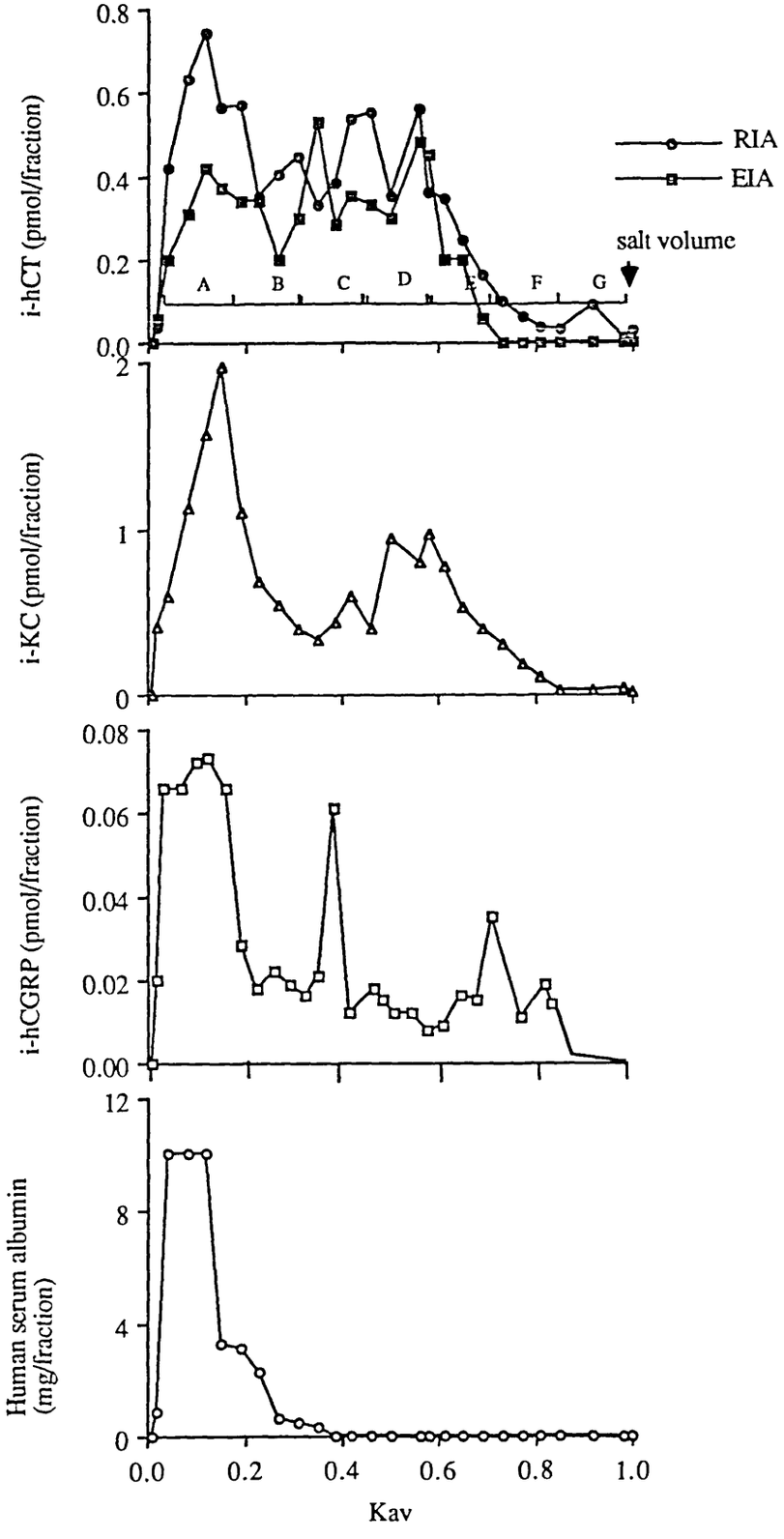


Figure 35: Elution profile of calcitonin gene peptides following gel-filtration of plasma from one patient with MTC

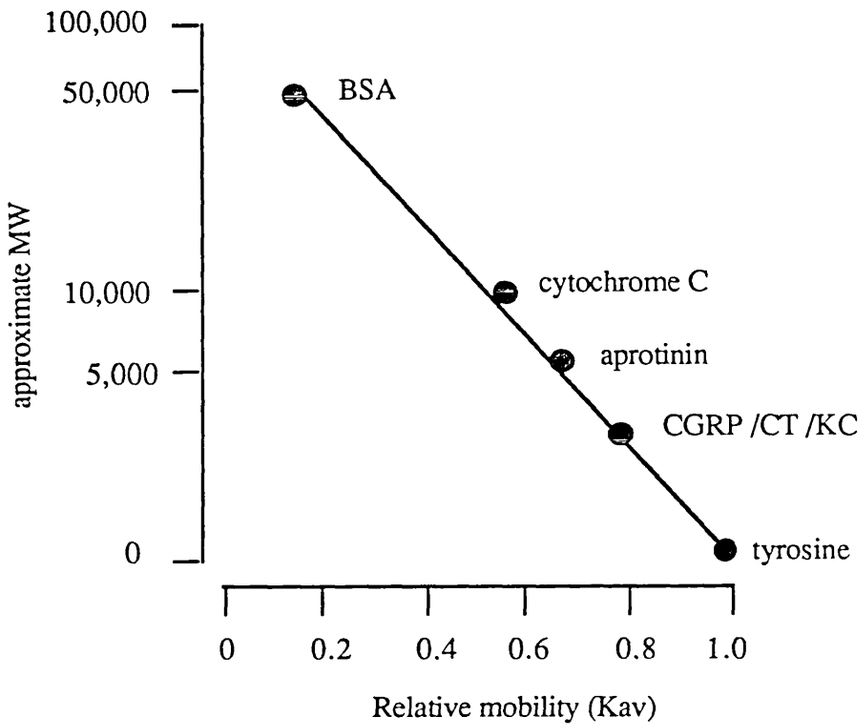


Figure 36: Calibration graph of the size-exclusion HPLC column (SW 2000) Relative mobility constant (Kav) was calculated from the following formula:

$$Kav = \frac{Ve-Vo}{Vt-Vo}$$

where Ve= elution volume, Vo=void volume and Vt=salt volume

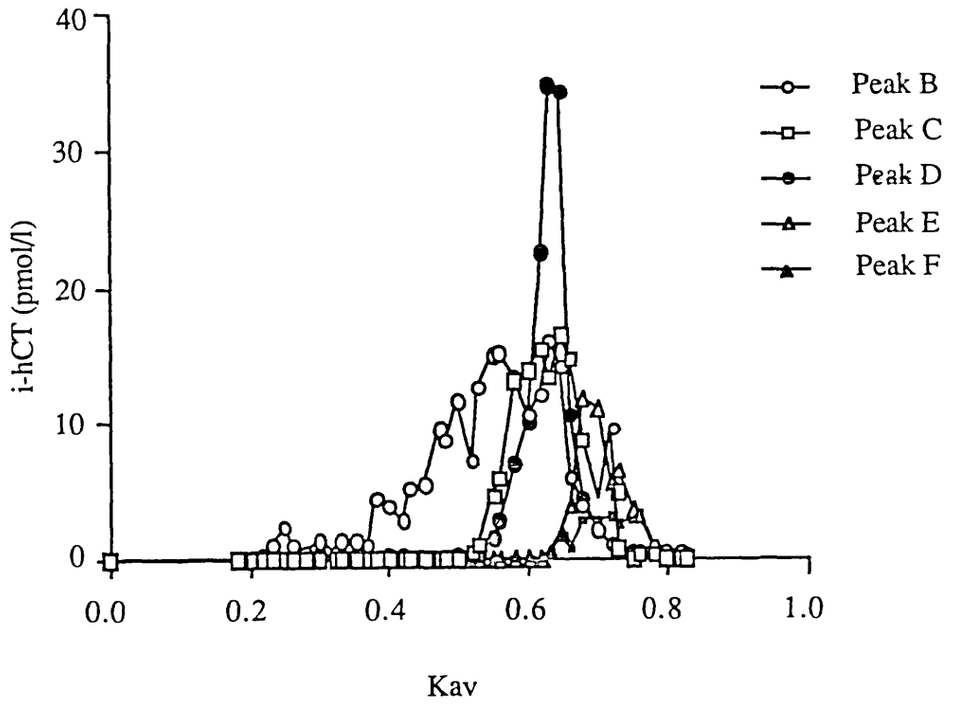


Figure 37: Elution profile of all i-hCT peaks as measured by RIA

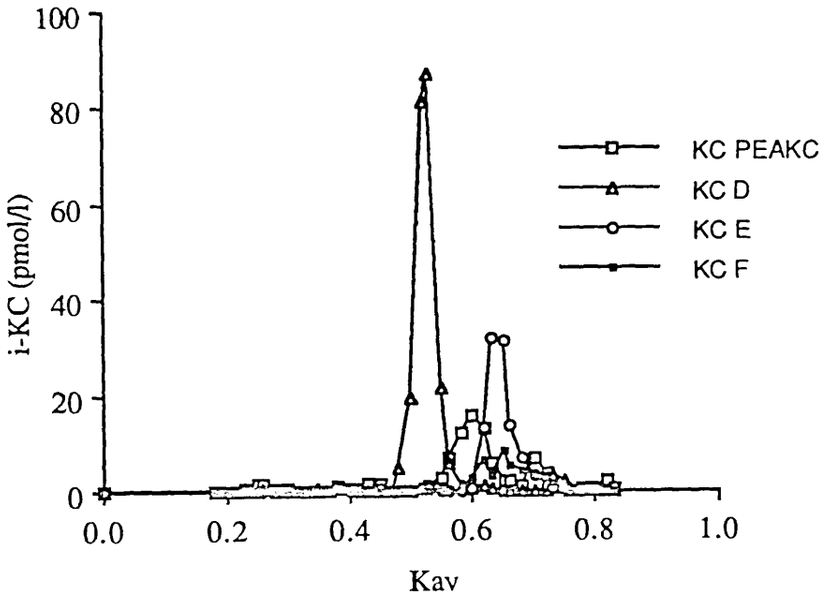


Figure 38: Elution profile of all i-KC peaks as measured by RIA

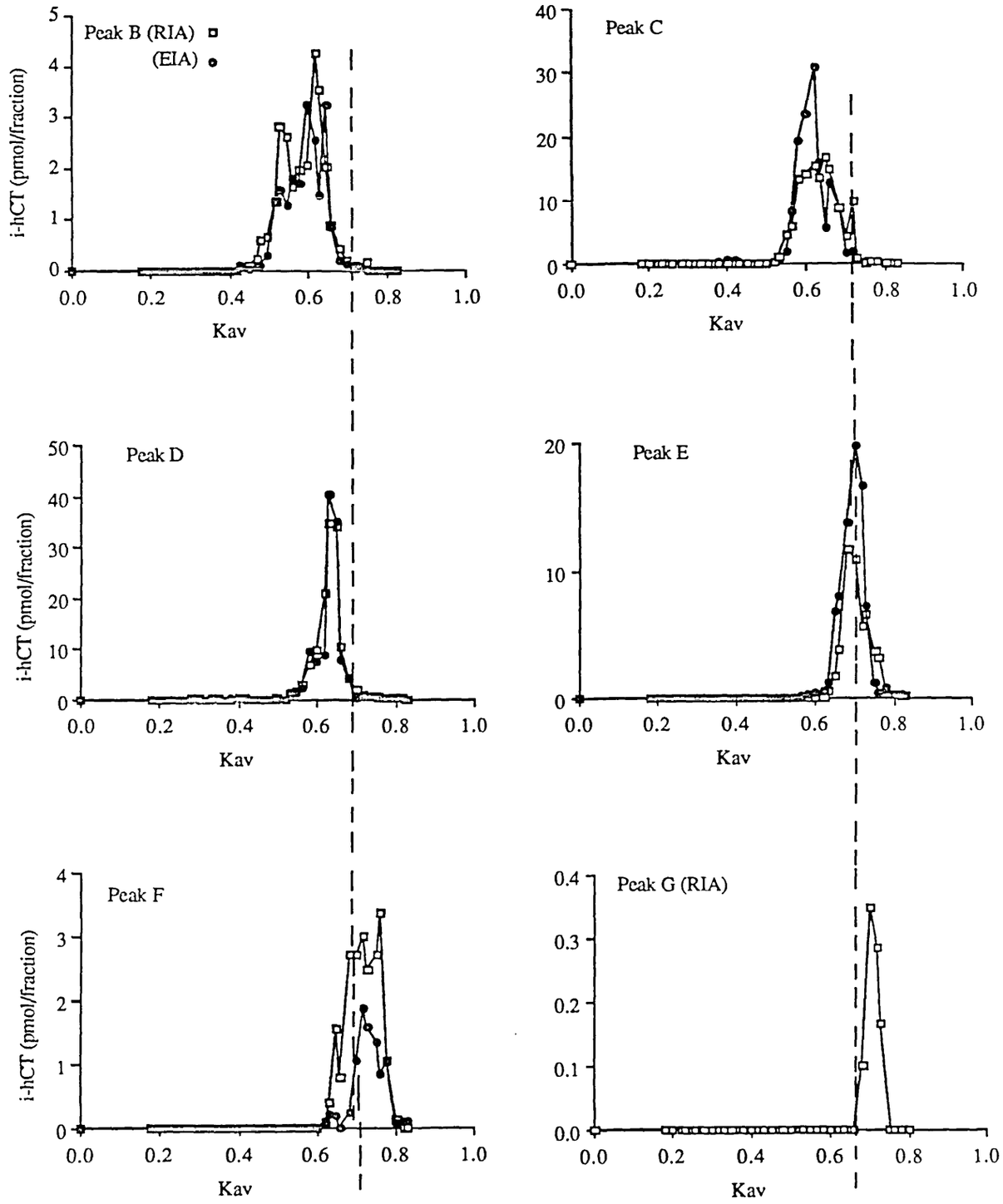


Figure 39: Size-exclusion high pressure liquid chromatogram (HPLC) of plasma from MTC patients. The dotted line indicates the elution position of the monomeric hCT

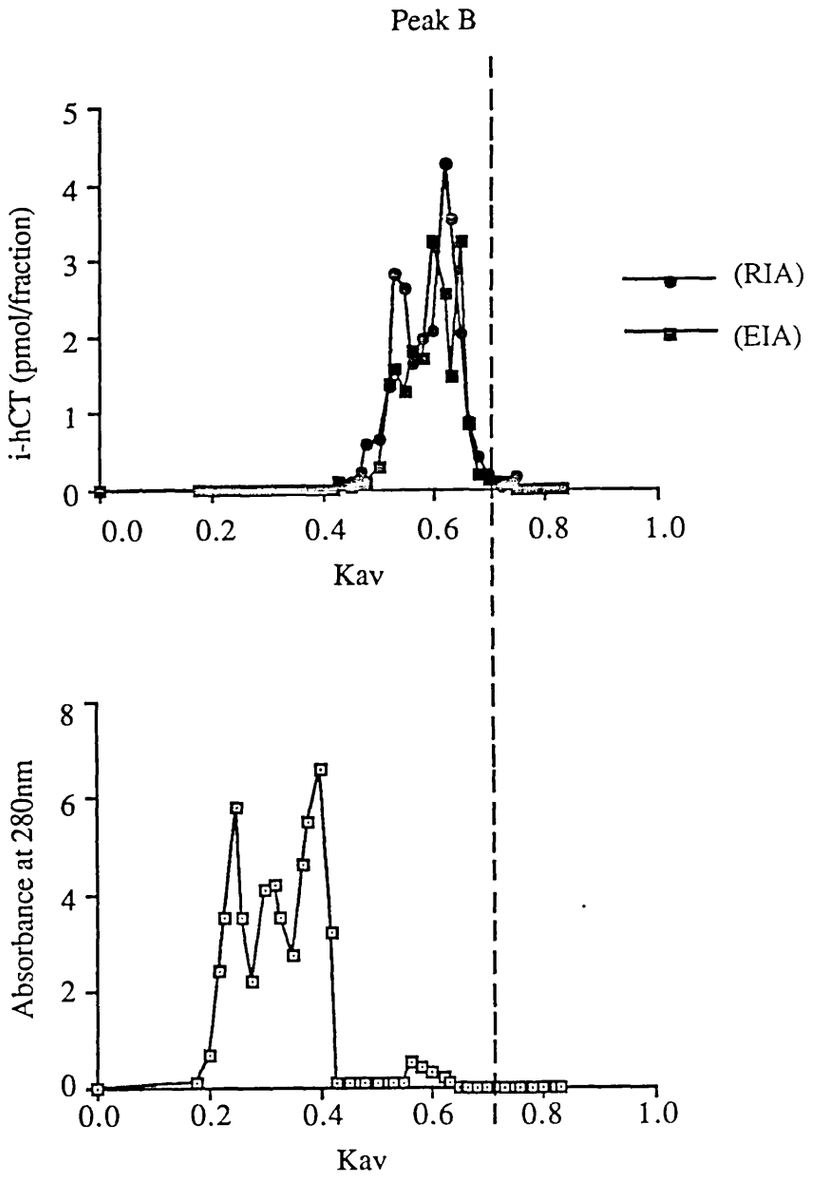


Figure 40: **Size-exclusion HPLC of peak B following gel-filtration of pooled plasma from patients with MTC. Dotted line indicates the elution position of radio-labelled hCT**

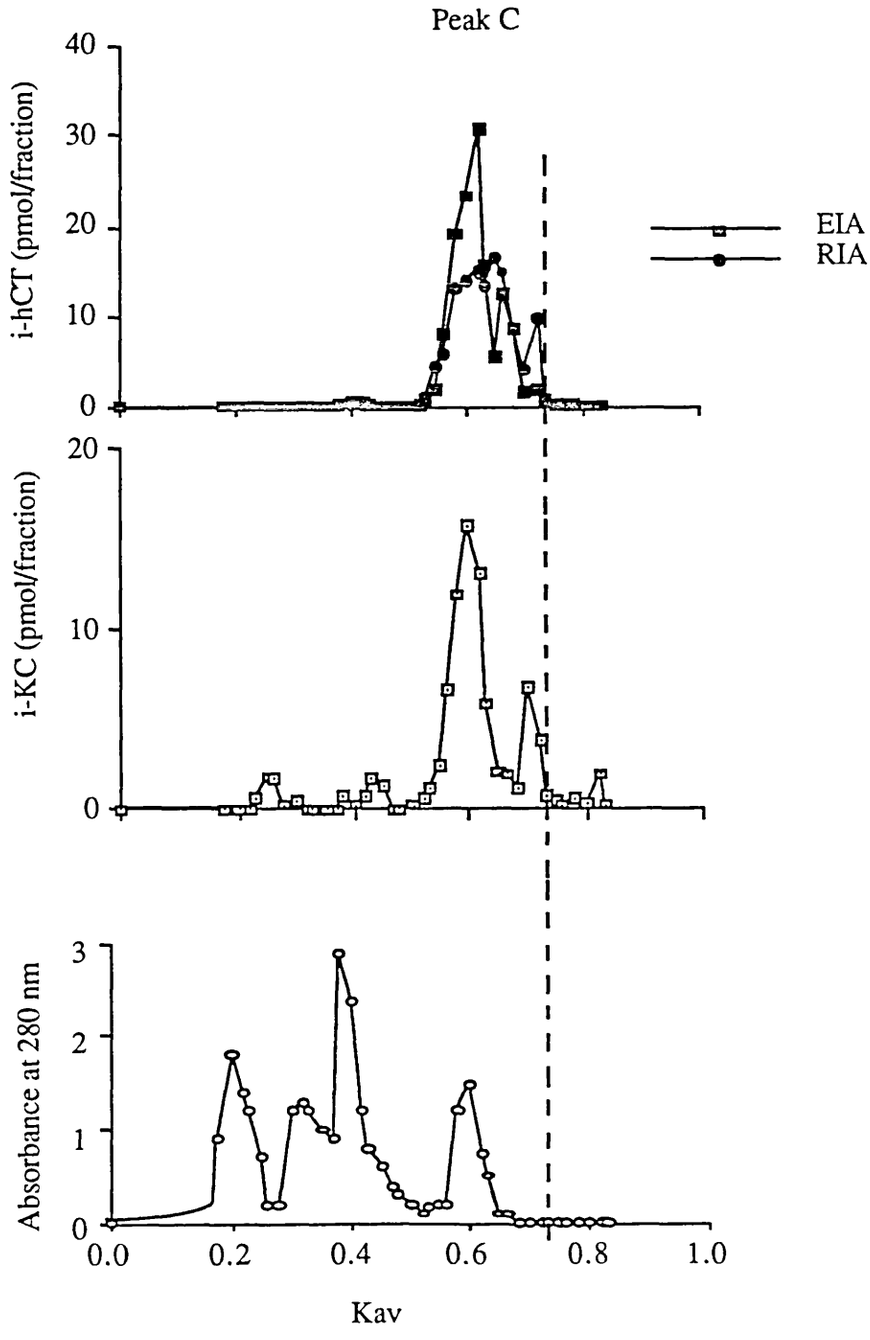


Figure 41: Size exclusion HPLC of peak C following gel-filtration of pooled plasma from patients with MTC. The dotted line indicates the elution position of radio-labelled hCT

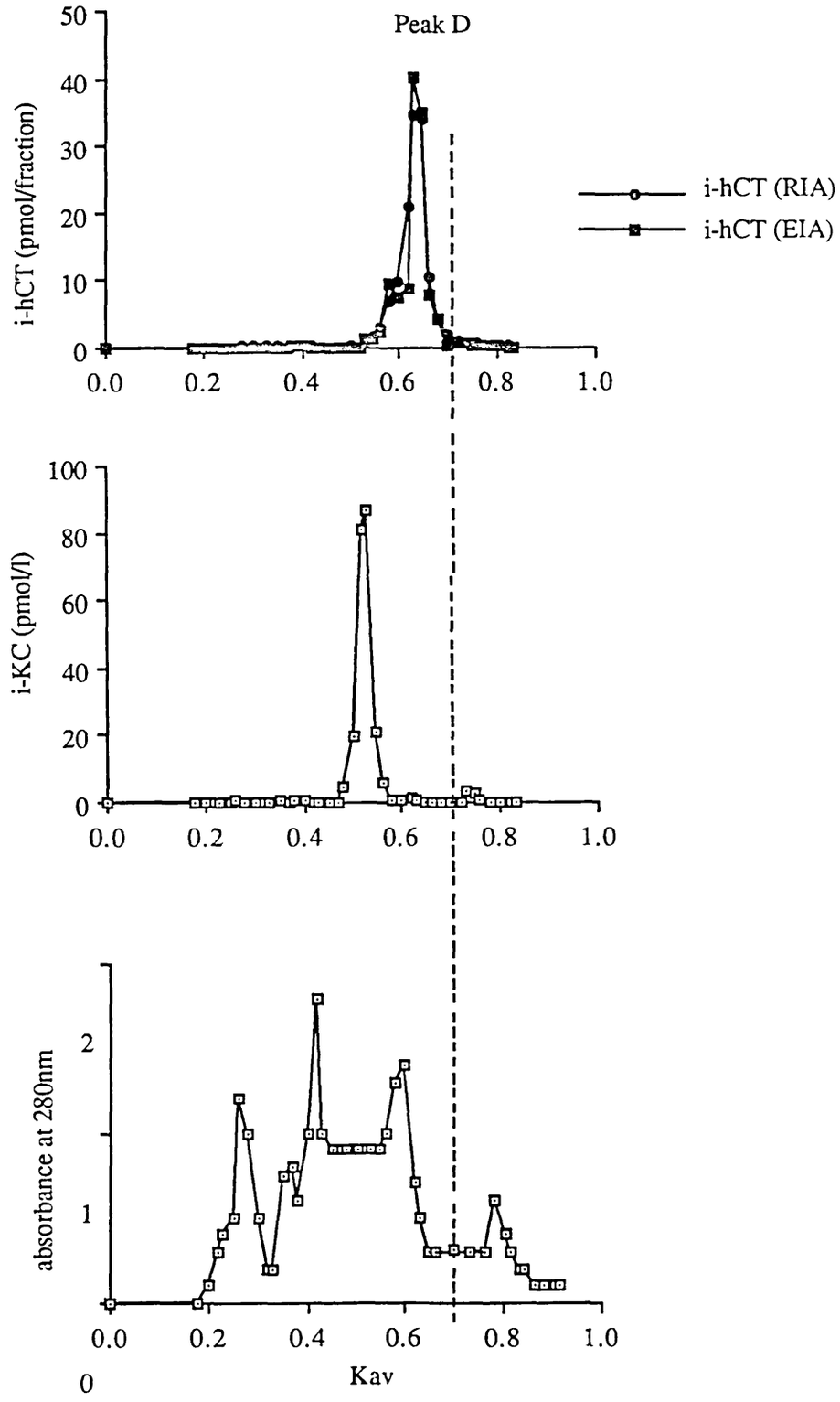


Figure 42: Size exclusion HPLC of peak D following gel-filtration of pooled plama from patients with MTC. Dotted line indicates elution position of radio-labelled hCT

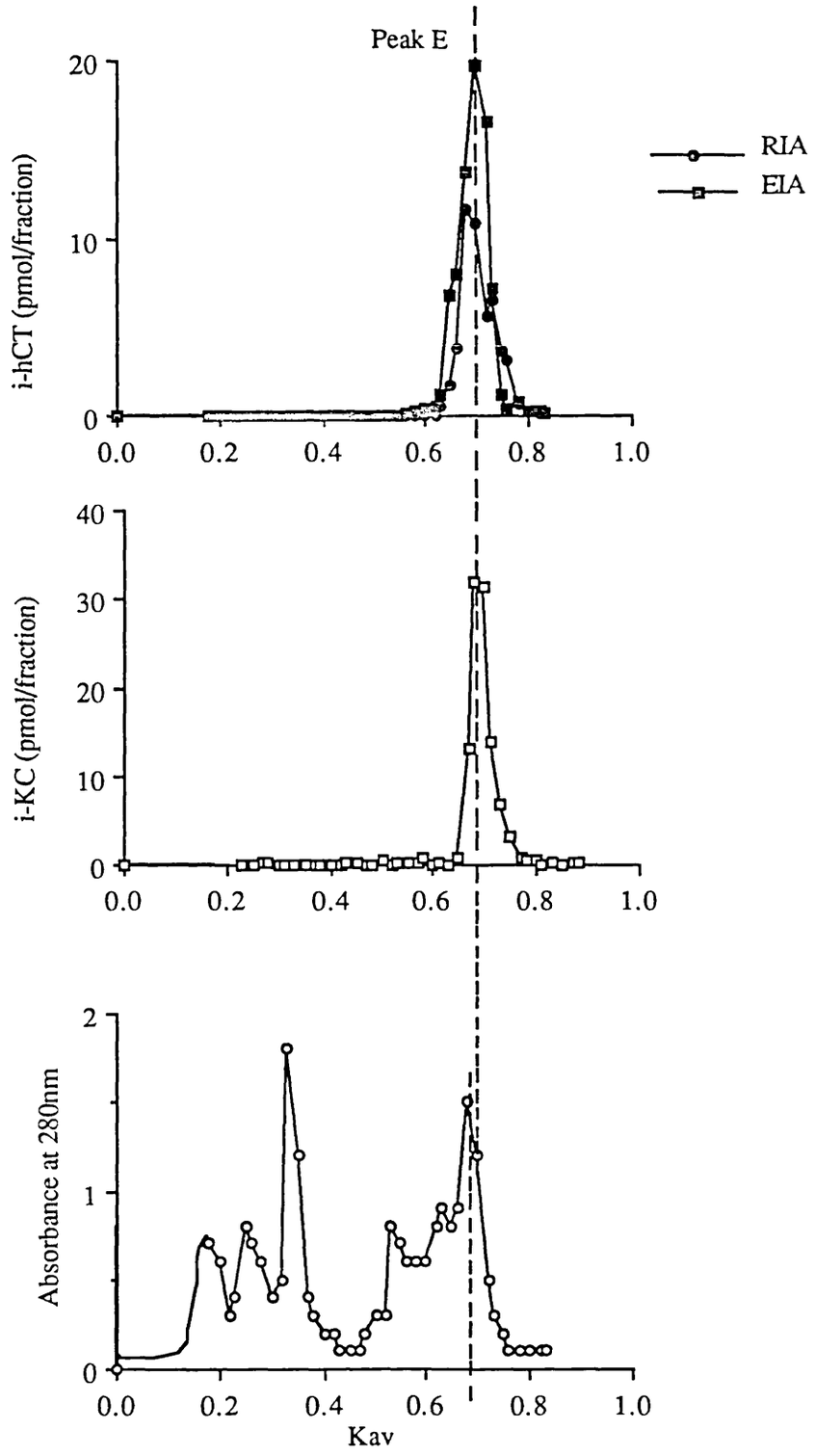


Figure 43: Size exclusion HPLC of peak E following gel-filtration of pooled plasma from patients with MTC. Dotted line indicates the elution position of radioactive hCT

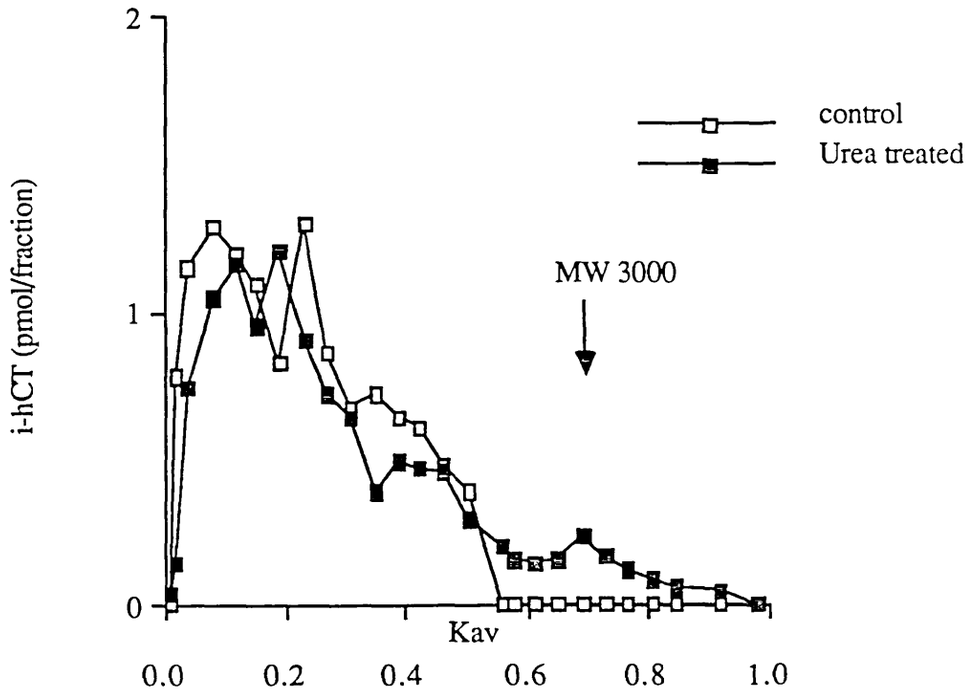


Figure 44: Elution profile of i-hCT from peak A (void volume peak)before and after treatment with urea followed by gel-filtration chromatography on spehadex G-50.

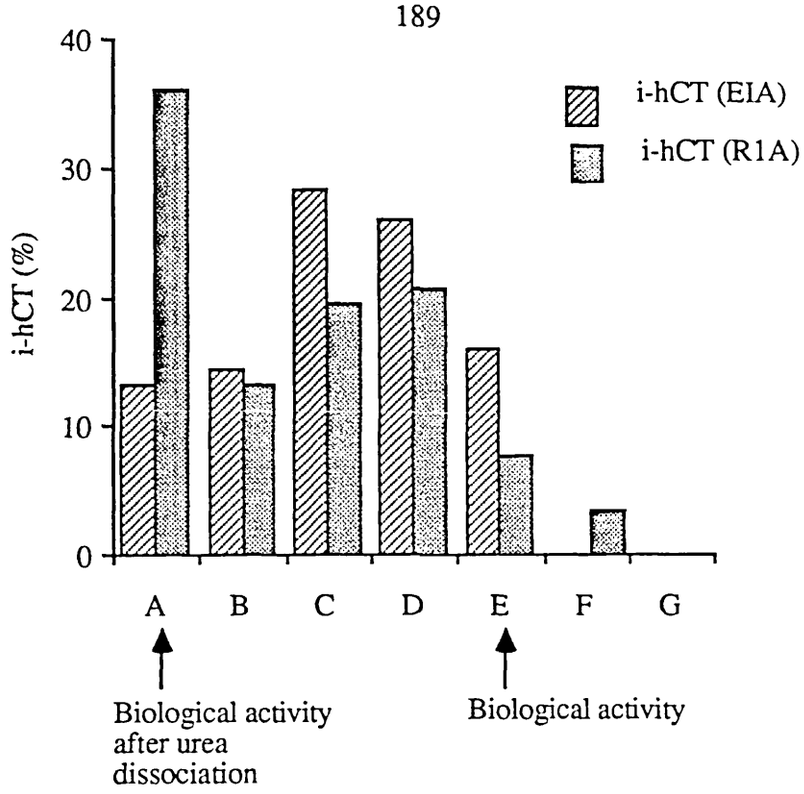


Figure 45: Comparision of the percentage of i-hCT in the various peaks from plasma of MTC patients using EIA and RIA

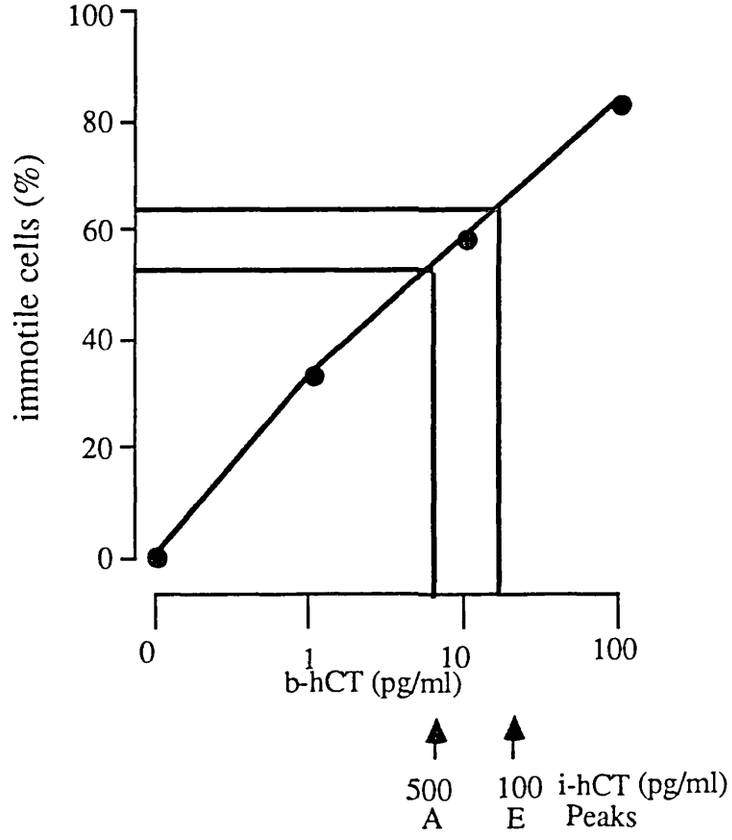


Figure 46: Calibration graph of isolated osteoclast bioassay. Arrows indicate the quantity of biologically and immunoreactive hCT in peaks A and E.

4.7. Discussion

Monoclonal antibodies employing non-isotopic markers can eliminate many problems commonly encountered with current assays which employ polyclonal antisera and isotopic labels.

The first production of monoclonal antibodies to hCT was reported in 1984 by Sturmer, Jones and Sternick. A two-site EIA for hCT using monoclonal antibodies has been reported recently (Berger, Shanes, Hart, Dissinger, Hill and Murayama, 1986). The sensitivity of such an assay was found to be 3.5 pmol/l. However there was no report on the specificity of these antibodies regarding epitope recognition .

The antibodies used in the present study have already been used to develop an immunoradiometric assay using the coated bead format. This involved adsorption of the monoclonal antibody CT07 (recognising the 26-32 amino acid region of the hCT molecule) on to the plastic bead as a solid-phase support. Antibody CT08 (specific for 11-17 region of the hCT molecule) was labelled with a radioisotope and added to the beads along with the samples and standards. At the end of the reaction, the beads were washed and the radioactivity incorporated was quantitated in a γ-counter. The sensitivity of this assay was reported as 3 pmol/l using a one-step, overnight incubation protocol.

In both the above methods, it seems that the ultimate sensitivity of these assays was limited not by the antibody characteristics but by the poor performance of the detection system. Hence, when enzyme amplification was employed using the same antibodies as the immunoradiometric assay, the detection limit of the assay was improved from 3 pmol/l to 1.7 pmol/l. An additional advantage of this approach was in the use of a non-isotopic label which did not decay throughout the course of this study.

The results show that the assay was specific for hCT and that the sensitivity of the overnight EIA was comparable with a 7 day incubation RIA. Both RIA and EIA confirmed grossly elevated i-hCT in all patients with MTC. The results indicated that both EIA and RIA recognised multiple forms of circulating hCT.

In normal individuals, a 7-day RIA was used to achieve adequate sensitivity. whereas a similar sensitivity was achieved using an overnight EIA.

The application of an enzyme amplification system in this study has indeed contributed significantly by increasing the sensitivity and the speed at which these assays can be carried out. In figure 33, a calibration graph for hCT which was obtained by performing a fast (20 minutes) assay for hCT is shown. The sensitivity of such an assay is reduced to 50 pmol/l but in a clinical sense, the speed of such an assay should prove very useful where critical decisions have to be made on the basis of circulating peptide levels for example, in pre-operative and post-treatment of MTC.

The plasma i-hCT levels of normal individuals and patients with MTC were lower when measured by EIA than those measured by RIA. It seemed likely that this was due to the specificity of the EIA in recognising only the "mature" form of the hormone. To test this theory, gel-filtration studies were carried out using pooled plasma from patients with MTC.

The results indicated that both EIA and RIA recognised multiple forms of circulating i-hCT. The main difference in the level was due to greater recognition of the i-hCT associated with high molecular weight protein (MW > 60,000) by RIA than by EIA. In addition, the circulating peptide fragments (indicated by peaks F and G) were not recognised by the EIA.

The identity of these larger molecular forms of i-hCT remains unknown, but it can be speculated that they may represent either unprocessed precursor forms of the hormone or glycosylated and non-glycosylated forms of the hormone which may have become aggregated (Roos *et al.*, 1983). Dissociation with urea was not performed on peaks B, C and D (see table 16)

The isolated osteoclast bioassay revealed that the monomeric form indicated by peak E was biologically active. However, there was a large discrepancy between the immunologically active hormone level and the biological activity present. The reason for this is not clear but it is likely that oxidation of the methionine residue at position 8, during chromatography has rendered the hCT

RIA

Total i-hCT 54 pmol	Void volume peak (peak A)	Monomeric peak (peak E)	Other peaks (peaks B,C, D, F &G)
Initial gel-filtration	19 pmol (35%)	4 pmol (8%)	31 pmol (71%)
i-hCT after urea treatment and re-chromatography of void volume peak	13 pmol (13/54 x100) = 24%	4 pmol (4/54 x100) = 7.4%	2 pmol
Total monomeric i-hCT by RIA		8% +7.4% = 15.4%	

EIA

Total i-hCT 40 pmol	Void volume peak (peak A)	Monomeric peak (peak E)	Other peaks (peaks B,C, D, F &G)
Initial gel-filtration (40 pmol)	5 pmol (13%)	6 pmol (16%)	29 pmol (71%)
i-hCT after urea treatment and re-chromatography of void volume peak	3 pmol (3/40 x100) = 7.5%	0.6 pmol (0.6/40 x100) = 1.5%	1.4 pmol
Total monomeric i-hCT by EIA		16% +1.5% = 17.5%	

Table 16: Summary of the percentage of the various forms of i-hCT, eluted after each chromatographic step, in pooled plasma from patients with MTC

molecule inactive since precautions were not taken to avoid oxidation. Earlier studies (Girgis, 1980) had shown that oxidation of the monomeric form of hCT to the sulphoxide form could occur during extraction and chromatography.

Some biologically active hormone can be released from the protein bound fraction indicated by peak A following urea dissociation. This observation was important since assays employing prior extraction of the monomeric peptide can lead to false interpretation of the total circulating peptide (Heath, Body and Fox, 1984)

In conclusion, these studies indicate at least by an *in vitro* bioassay, that the larger immunochemical forms of calcitonin representing the majority of the total immunoreactive hCT in circulation of MTC patients are biologically inactive.

Chapter 5: General discussion and future trends

Chapter 5: General discussion and future trends

In this thesis, I have described the development and applications of non-isotopic immunoassays for two calcitonin gene peptides, namely calcitonin and CGRP. The advantages of these assays over the conventional RIA were discussed. The main technical problems were the limited half-life of the radioactive tracers, the lack of specificity of the polyclonal antibodies towards a particular circulating form of the peptide and the sensitivity of the detection system. The main physiological problems which existed with the measurement of CT and CGRP, as with other peptide hormones, were the immunochemical heterogeneity of the circulating peptides and their presence in a very low concentration in the plasma of normal individuals (Body and Heath, 1983). Therefore, the main disadvantages of the conventional RIA's were that long incubation times were employed to achieve the desirable sensitivity. This could cause proteolytic degradation of the peptides by the endogenous enzymes present in the plasma samples. The consequence of all these factors is that conventional RIA's even when carried out in highly competent laboratories often yield markedly different results.

It was possible to improve the detection limit of these assays by applying the method of enzyme amplification. In the one-site assay for hCT employing polyclonal antiserum and enzyme amplification method as the detection system, it was possible to use a very low quantity of an enzyme-labelled hCT thereby increasing the sensitivity of the resulting assay to a point where it out performed the best RIA for hCT which used the same antiserum (Lynch, Seth, Bates and Self, 1988).

The application of the enzyme amplification has contributed significantly to not only increasing the sensitivity but also the speed at which these assays can be carried out. A fast assay could be carried out in 20 minutes. The speed of such an assay may prove very useful where critical decisions have to be made on the basis of circulating peptide levels in pre-operative and post-treatment of

MTC. It will be useful in identifying patients with the familial form of MTC where the disease may not be apparent clinically but following provocative tests with CT secretagogues, the i-hCT levels become highly elevated. This test may be carried out in an out-patient clinic where the results of the test can be readily obtained within a very short time.

Ideally when MTC is diagnosed, total thyroidectomy should be performed. The most important and useful application of a fast assay would be in rare cases where there is recurrence of the tumor and the surgeon would like to pin-point the site of malignancy by measuring i-hCT peri-operatively (in the blood vessels draining out of the thyroid gland). This may provide surgeons who manage these patients with an unusual opportunity to control the disease by removing the affected tissue efficiently. The added advantage of using an enzyme amplification system is that since a stable non-isotopic label is used, it allows construction of assays which may be read by simple spectrophotometric means or merely by visual inspection of the formazan colour (see plate 5).

The specificity of the peptide assays was improved by changing the format of the assays from one-site to two-site (immunometric). The advantages of the two-site assays have been summarised in chapter 2. Using well characterised polyclonal antibodies (as with rat CGRP assay {Seth, Zaidi, Fuller and Self, 1988}) the two-site assay appeared to be more specific for the whole molecule than the comparable RIA. This finding was expected in view of the fact that different antibodies each reacting with a different region of the CGRP molecule had been used. The overall high performance of this assay was somewhat surprising. It is likely that this assay could be improved further, as in the study carried out on a similarly small peptide ACTH (Hodgkinson *et al.*, 1984), by immunopurification using synthetic hCGRP sub-peptides to isolate two antibodies which recognise spatially distinct and different epitopes since high titre monoclonal antibodies against hCGRP have not been obtained so far.

The monoclonal antibody-based enzyme immunometric assay for hCT had been strategically designed in such a way that it was specific for the "mature" form of the hormone. This meant that any circulating calcitonin which forms part

of a precursor, and therefore does not contain the amidated proline residue, was not detected by the solid-phase antibody CT07 since it was specific for 26-32 amide containing region of hCT molecule.

Using this assay, previous findings that i-hCT levels in MTC are grossly elevated and that in normal subjects, women have lower CT levels than men were confirmed (Seth, Motte, Kehely, *et al.*, 1988) . There was no significant difference in the circulating i-hCT levels of caucasians and the negroid populations.

Human CT assays are becoming relevant in the field of osteoporosis where the levels of the hormone have been shown to be lower in post-menopausal women. It is possible that the measurement of plasma hCT may prove to be a marker that identifies those women most at risk of losing bone rapidly. Therapy using calcitonin has been shown to be as effective as oestradiol in the prevention of post-menopausal bone loss (MacIntyre *et al.*, 1988). There is a clear need for a sensitive assay which can detect low levels of i-hCT in a population in which the levels are at lower end of the reference range. This particular assay may prove useful in this respect and further studies are underway.

From the immunochemical studies following gel-filtration chromatography (chapter 4), it can be speculated that hCT is secreted in a monomeric form from the C-cells in the thyroid. It becomes associated with high molecular weight proteins such as albumin and circulates in this form. The albumin-associated hCT (peak A) is recognised by both the RIA and the more specific EIA (although to a much lesser extent). The metabolic product includes fragments which can be detected in the plasma or urine of normal subjects as well as patients with MTC. In the present study, fragments have been detected by RIA but not the specific EIA.

It is interesting to note that in patients with MTC, grossly elevated circulating i-hCT levels are tolerated without exhibiting hypocalcaemia (Hill, Ibanez, Samaan, Ahearn and Clark, 1973; Austin and Heath, 1981). This supports the theory that monomeric hCT secreted from the thyroid can become associated with a high molecular weight protein thereby rendering it inactive, or that a down regulation of CT receptors occurs with persistent stimulation in analogy

with the *in vitro* 'escape' phenomenon (Raize, Au, Friedman and Neimann, 1967; Tashjian *et al.*, 1978) and *in vivo* tachyphylaxis (Obie and Cooper, 1979) observed for CT.

Certain peptide hormones may exist in Asn-linked glucosamine-containing glycosylated forms. Examples of such hormones are ACTH, prolactin and CT. The effect of glycosylation on the biological activity of peptide hormones is not well understood but it appears to depend on the location of the oligosaccharide. Glycosylation alters prolactin but not ACTH bioactivity depending on the location of the oligosaccharide.

Calcitonin is glycosylated at the asparagine residue on position 3, a position thought to be critical for biological activity. A study has been carried out recently to test the effect of glycosylation on CT bioactivity (Gkonos, Lawson, Howard, Walsh, Birnbaum and Roos, 1988). The authors isolated glycosylated-CT from a rat MTC tumor and tested its biological activity on young rats. They did not observe a decline in serum calcium and concluded that glycosylated CT is not biologically active.

In the present study, the identity of peaks C and D remain unknown, but it can be speculated that one of them (probably peak D) may represent the glycosylated form of the mature hormone since glycosylated CT has a sequence identical to CT (Gkonos *et al.*, 1988). Peak C probably represents the aggregated (polymer) form of glycosylated and/or non-glycosylated hCT since it elutes at a higher molecular weight position than the monomeric form. Both the above peaks were found to be biologically inactive on the isolated osteoclast bioassay system. It is unlikely that they represent unprocessed precursors, since they are being recognised by the specific EIA.

The monomeric form indicated by peak E was found to be biologically active. However, there was a large discrepancy between the immunological and the biological activity present. Earlier studies (Girgis, 1980) had shown that the monomeric form of hCT can be readily oxidised to the sulphoxide form during extraction and chromatography. It is possible that in the present study, partial

oxidation of the methionine residue at position 8 on the hCT molecule has rendered it partly inactive since precautions to avoid oxidation were not taken during gel-filtration.

The present study has shown that even a highly specific immunoassay will recognise polymers and protein-associated forms of the peptide hormone. In future, efforts need to be put in developing receptor-based assays, such as that developed for CGRP (Wimalawansa , personal communication), since they probe for high affinity binding of hormonal peptides and not, as in an immunoassay, for antigenic determinants which may be present not only on the native hormone but also on polymers, precursors and fragments. Specific, high affinity receptors for calcitonin have been identified in human placental membranes (Nicholson, D'Santos, Evans, Moseley, Kemp and Martin, 1988) and it may be possible to use this preparation to develop a receptor based assay for hCT in the near future.

Advances in molecular biological techniques have contributed significantly to the study of the expression of these peptides at the genomic level. The recent technique of *in situ* hybridisation of labelled complementary DNA (cDNA) probes to specific mRNA or DNA sequences in cells and tissues has become a valuable morphological tool. By using this technique in conjunction with immunocytochemistry using specific monoclonal antibodies, simultaneous detection of both gene message and gene product at the cellular level can add a new dimension to the study of medullary thyroid carcinoma and the tumor products particularly, the calcitonin gene products.

Finally, the main conclusions that can be drawn from this study are:

*It is possible to improve existing conventional immunoassays by applying a more sensitive detection system such as enzyme amplification.

*By using existing polyclonal antisera, specific for different parts of molecule, it is possible to construct immunometric assays which are superior to the conventional RIA in terms of specificity.

*Using the monoclonal antibody-based enzyme immunometric assay developed in this study which is the most sensitive assay for i-hCT to date, earlier findings regarding CT levels in women have been confirmed.

*Gel-filtration studies in conjunction with assessment of the biological activity of i-hCT from MTC plasma showed for the first time that part of the protein-associated CT is potentially biologically active. Therefore, assays based on prior extraction of the monomeric hCT (Heath, Body and Fox, 1984) will underestimate levels of circulating, biologically active hormone.

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Publications

JOURNAL OF IMMUNOASSAY, 9(2), 179-192 (1988)

CALCITONIN DETERMINATION BY A FAST AND HIGHLY SENSITIVE ENZYME AMPLIFIED IMMUNOASSAY

Catherine Lynch, Rashmi Seth, David L. Bates*, Colin H. Self

Department of Chemical Pathology,
Royal Postgraduate Medical School,
Hammersmith Hospital,
DuCane Road, London W12 OHS.

* IQ (Bio) Ltd.,
Downham House, Downhams Lane,
Milton Road, Cambridge CB4 1XG.

Key words: Enzyme amplification - enzyme immunoassay - calcitonin - medullary carcinoma - thyroid

ABSTRACT

A colorimetric enzyme amplification system was used to develop an immunoassay for human calcitonin (hCT) with a sensitivity of 6 pmol/l, and intra- and inter-assay CVs of 12% and 11.8% respectively for the low pool, and 10% and 11.2% for the high pool. The mean recovery of added synthetic hCT (58.5 pmol) from the plasma of 10 patients was 110% (64.4 pmol). The correlation coefficient between radioimmunoassay (RIA) and amplified enzymo-immunoassay was found to be 0.96 (p 0.001). The assay was successfully applied to the measurement of elevated

Reprint requests to:

Rashmi Seth,
Department of Chemical Pathology,
Royal Postgraduate Medical School,
London W12 OHS, U.K.

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calcitonin levels in plasma from patients with medullary carcinoma of the thyroid (MCT). AEIA offered a reliable and sensitive alternative to RIA for calcitonin determination with the added advantage of convenience as the label employed was much more stable.

INTRODUCTION

Initially bioassays were used to measure human calcitonin in plasma from patients with medullary carcinoma of the thyroid (MCT) (1). However, the extraction of very large volumes of plasma was required to show the presence of hCT in normal adult plasma (2). Later, a radioimmunoassay (RIA) was developed (3) which was more specific and less laborious. Through these approaches the diagnostic value of hCT determinations is now well established and high risk individuals are screened for MCT and the progress of the disease followed by such a method (4). In addition, a variety of non-thyroid malignancies have also been associated with elevated levels of plasma hCT (5,6,7).

Whilst RIA has been indispensable in demonstrating the clinical value of hCT measurements, the disadvantages of using RIAs for hCT estimations are that long incubation times are necessary for the sensitive assays which are required to measure normal levels. Freshly labelled reagents have to be prepared frequently owing to the relatively fast decay of the radioactive label. There is, therefore, a need for a robust, sensitive and fast assay with reproducibly high precision utilising a stable label. We have developed such a method for calcitonin by

application of enzyme amplification as previously described (8,9).

The assay format was established as being competitive to provide a direct comparison to our competitive RIA. In such assays it is widely recognised that sensitivity is directly related to the affinity of the antibody used and inversely related to the amount of conjugated label which has to be employed to provide a quantifiable signal. Our intention has been to exploit the high signal generating power of enzyme amplification, thereby reducing the amount of enzyme conjugate employed in the assay and thus increase the sensitivity of the assay. The RIA used for comparison was set up to provide the highest sensitivity possible with the same antibody.

The particular amplification scheme employed here uses the enzyme alkaline phosphatase which is conjugated to synthetic hCT and competes with hCT in the sample for a surface bound antibody against hCT. The conjugate which successfully competes and becomes surface bound is then determined by its ability to dephosphorylate NADP to NAD which then activates a highly NAD-specific redox cycle to produce an intense colour (Fig. 1).

MATERIALS AND METHODS

Diaphorase (NADH:Lipoamide oxidoreductase (E.C. 1.6.4.3)) was obtained from Boehringer Corporation London; alcohol

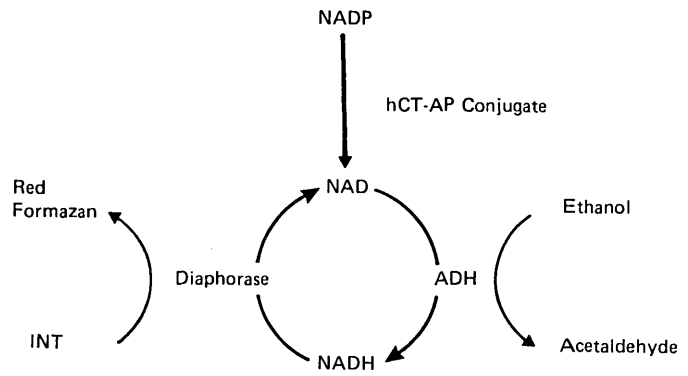


Figure 1

Diagram of enzyme amplification cycle triggered by alkaline phosphatase-calcitonin (AP-hCT) conjugate.

dehydrogenase (Alcohol NAD oxidoreductase (E.C. 1.1.1.1; ADH)) and p-iodonitrotetrazolium violet (INT) were obtained from Sigma Chemical Company; Chloramine-T was obtained from BDH Chemicals Limited. Sheep anti-rabbit γ-globulin was obtained from IRE-UK Ltd.; ^{125}I was obtained from Amersham and synthetic human calcitonin (hCT) was a generous gift from Dr. W. Rittel, Ciba-Geigy Ltd., Basle, Switzerland. Trasylol (aprotinin proteinase inhibitor) 20,000 kIU l^{-1}, was obtained from Bayer, Berks., UK and human plasma protein fraction B.P. from the Blood Products Laboratory, Herts.,U.K. All other reagents were of the highest grade available.

Antiserum

Antiserum to human calcitonin (hCT) was raised by repeated immunization of 3 rabbits with an hCT-ovalbumin conjugate. A primary injection of 100 µg/ rabbit CT in Freund's complete adjuvant was administered into the hind footpads of each rabbit. dorsal booster injections of 25 g hCT conjugate in Freund's incomplete adjuvant were given subcutaneously six weeks after the primary injections and then at monthly intervals until a satisfactory titre of anti-calcitonin antiserum was obtained (designated OC 31).

Plate-coating

Micro-titre plates from Nunc, Denmark, were coated with anti-calcitonin antiserum in 20 mM carbonate buffer, pH 9.6. To each well was added 120 µl of a 1:40,000 dilution of antiserum. The plates were sealed using Titertek Plate Sealers (Flow Laboratories Ltd) and incubated overnight at 22°C. The antibody solution was then shaken out and the wells washed four times with a buffer containing 5% lactose, 0.5% bovine serum albumin (BSA) and 0.02% Tween 20 (pH 7.4). The plates were dried, covered with plate sealer and stored in a desiccator at room temperature.

The CT-alkaline phosphatase conjugate was prepared by reducing the N-terminal disulphide bridge of hCT and coupling the resulting thiols to maleimide-activated alkaline phosphatase as follows. Human CT was dissolved in 0.1M triethanolamine-HCl (pH 8.5) containing 0.1M NaCl and 1mM EDTA. Dithiothreitol, 10 µl of

a 50 mM solution in water, was then added and the reaction incubated at 37°C for 1 hour. The reduced peptide was recovered by gel-filtration on a Sephadex G-10 column in 0.1M triethanolamine-HCl (pH 7.3) containg 0.1M NaCl and 1 mM EDTA. Calf-intestinal alkaline phosphatase (2mg) was reacted with succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate for 15 minutes and the reduced peptide and activated enzyme coupled and recovered as described previously (10).

Amplified Enzyme Immunoassay (AEIA)

Calcitonin standards were made by serial dilution of a concentrated standard in plasma protein fraction B.P. Samples and standards (80 µl) were placed in the wells of a micro-titre plate coated with anti-hCT antibody. The plate was covered and incubated overnight at room temperature. Twenty µl of conjugate (1:5000 initial dilution) was added to each well and incubated for 6 hours at room temperature. The samples were then shaken out and the wells washed four times with 50 mM Tris (pH 7.4) containing 0.15 M NaCl and 0.02% Tween 20.

A substrate solution (100 µl of 0.2 mM NADP in 50 mM diethanolamine at pH 9.5) was then added to each well and incubated at room temperature for 15 minutes. Following this, 200 µl of an amplifier solution consisting of 60 U/ml ADH, 0.1 U/ml Diaphorase, 0.55 mM INT and 4% v/v ethanol in 25 mM phosphate buffer at pH 7.0 was mixed into the contents of each well. The

reaction was allowed to proceed until the colour of the zero standard wells reached about 2 absorbance units at 492nm (approximately 20 mins). Sulphuric acid (50 µl of 0.2 M) was then added to each well and the absorbance of the wells read at 492 nm in a Titertek Multiskan MCC (Flow Laboratories).

Radioimmunoassay

Radio-iodination of synthetic hCT was performed by the Chloramine-T method (11) and the resulting label purified on HPLC using a linear acetonitrile/water/0.1% TFA gradient, from 25% to 45% ACN. The RIA method used was a modification of the previously published method (12) utilising antiserum OC31. The same antiserum was used in the amplified enzyme immunoassay. Synthetic hCT house standard was standardised against human calcitonin (Ref. 70/234) from NIBSC, Holly Hill, London. Serial dilutions of hCT were prepared in PPF and duplicate 100 µl of sample or standard were then dispensed with 100 µl of phosphate buffer (0.05 M, pH 7.4, containing 0.025% HSA and Trasylol). Antiserum OC 31 (50 µl in phosphate buffer) was added to each tube to give a final dilution of 1 in 180 000 in the assay tube. Fifty µl of ^{125}I hCT were then added to each sample to give 10 000 counts in 100 seconds. Following an overnight incubation at 4°C the assay was separated using sheep anti-rabbit second antibody bound to cellulose.

Coefficient of Variation

To determine the precision of the AEIA two quality control

pools were prepared at approximately 73.1 pmol/l and 292.6 pmol/l hCT. Each pool was assayed several times using different wells on single plates and then repeatedly in 10 separate assays using different plates to obtain intra-plate and inter-plate coefficient of variation (CV).

Recovery

Plasma samples were obtained from 10 patients with non-thyroid malignancies. Synthetic hCT was added to a 1 ml volume of plasma from each patient to give a final concentration of 58.5 pmol/l. The plasmas, before and after addition of synthetic hCT, were then assayed using the AEIA and the mean percentage recovery of hCT determined.

Correlation with clinical samples

Samples of plasma from various patients with MCT and non-thyroid malignancies were assayed by both RIA and AEIA. The results obtained were used to determine the correlation between RIA and AEIA.

RESULTS

A standard curve for the competitive calcitonin AEIA is shown in Fig. 2, which shows the overnight assay to have a sensitivity of 6 pmol/l. For comparison the figure also shows a standard curve for calcitonin measured by RIA using an overnight incubation and employing the same antibody. The AEIA is more sensitive.

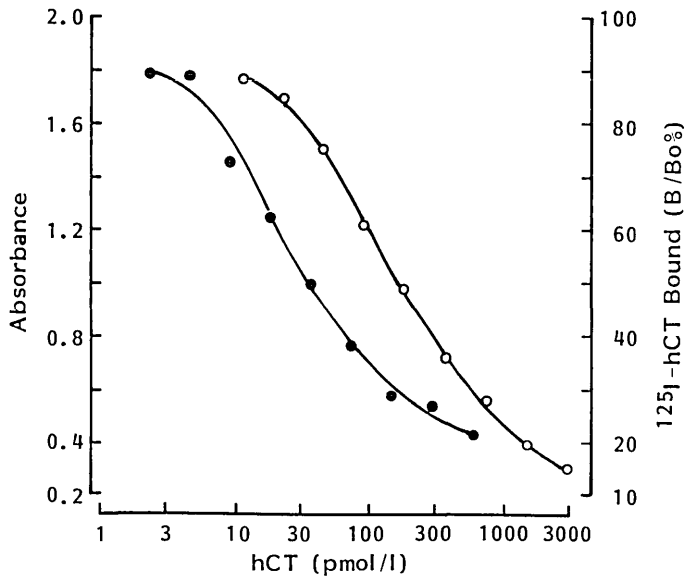


Figure 2

Calcitonin standard curves determined by AEIA (●) and RIA (○).

Coefficient of Variation

The within assay CV of the lower pool (mean 61.4 pmol/l) was found to be 12% (for 16 replicates). For the higher pool (mean 307.2 pmol/l) the intra-plate CV was 10%. Using the same pools the inter-plate CV for 10 assays was 11.8% for the low pool and 11.2% for the high pool.

Recovery

The mean recovery of 58.5 pmol/l added synthetic hCT from the plasma of 10 patients was 64.4 pmol/l (110%).

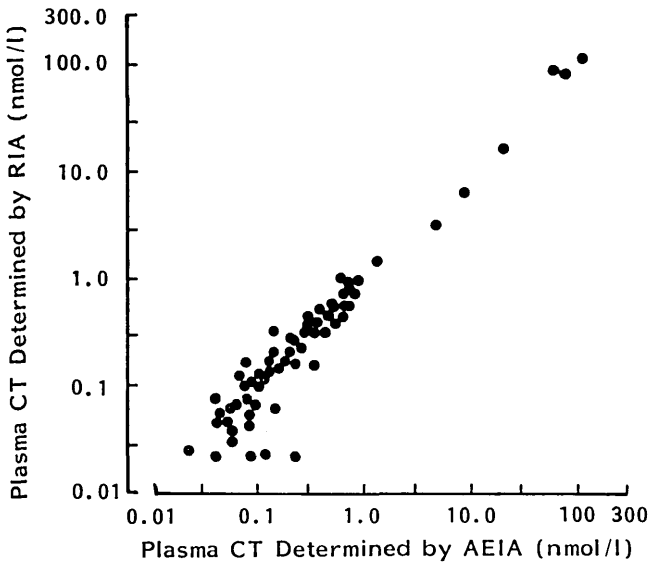


Figure 3

Correlation of plasma samples determined by RIA and amplified enzyme immunoassay. Samples were obtained from patients with MCT and non-thyroid malignancies.

Correlation with clinical samples

The correlation between the RIA and AEIA is shown in Figure 3. The correlation coefficient (v) was found to be 0.96 (p 0.001) and the regression equation was y= -0.0776 + 1.02x. Fig. 4 shows the dilution of hCT in plasma from patients with medullary thyroid carcinoma to be parallel to the standard curve.

DISCUSSION

The method of choice for hCT measurement has long been RIA.

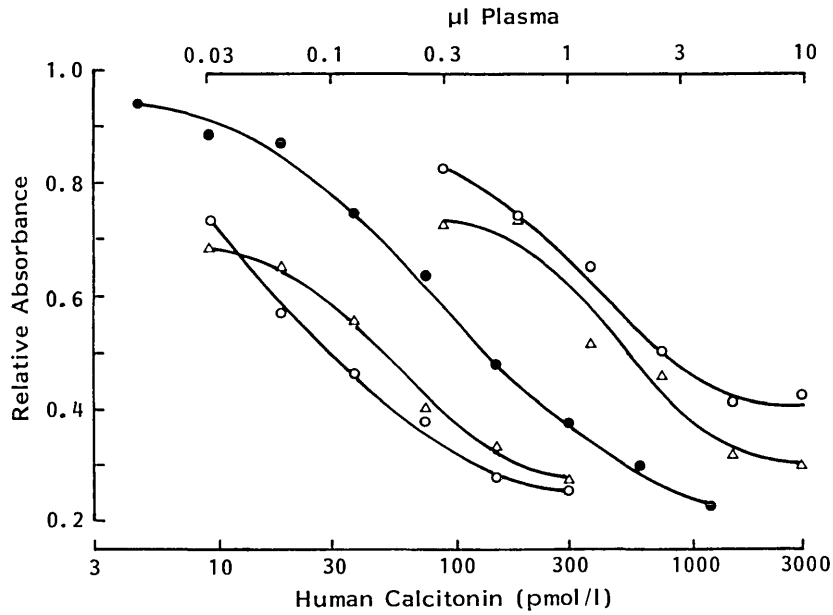


Figure 4

Calcitonin standard curve (●) (n = 6) showing parallel dilutions of immunoreactive calcitonin in plasma from patients with MCT (○ , △).

The limitations of RIAs are the instability of the radiolabel, long counting times, the fact that a scintillation counter is required and the health hazards and disposal problems of ionizing radiation.

We have found amplified enzyme immunoassay to offer a reliable and sensitive alternative to RIA in the determination of calcitonin. The increased signal produced in the assay by enzyme

amplification has allowed us to employ very low concentrations of conjugate and increase the sensitivity of the assay to a point where it outperforms the best comparable RIA we have been able to develop using the same antiserum. The assay is also more convenient in that the enzyme label is much more stable than an ^{125}I label, particularly when stored at -20°C. The microtitre plate format we have used adds to the convenience in that samples may be easily loaded onto the plates and the developed plates read quickly using readily available plate readers. It must, however, be emphasised that such amplified assays depend greatly on a good separation of specific signal from noise. In this respect it is important to achieve good antibody plate coating and in particular care must be exercised with respect to obtaining an even coating of wells on the edge of the plate.

In a clinical sense the speed and sensitivity of the assay should prove useful. In screening for medullary carcinoma of the thyroid where the expected levels of calcitonin are high (4) the results may be obtained in a few hours. Conversely, in the assay of plasma from patients with non-thyroidal malignancies, and expected calcitionin levels near the normal range, the fact that the assay is capable of determining calcitonin levels within the normal range without long incubation periods or extraction procedures is important. In addition to these clinical uses we hope that enzyme amplified immunoassay will be of value in the further elucidation of the physiological role of calcitonin and

thereby remove some of the controversy which presently exists (13).

Acknowledgement

We are very grateful to the Leukaemia Research Fund and the MRC who have supported this work by grants to CL and RS. We thank Dr PJR Bevis for the radiolabelling of hCT.

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Journal of Immunological Methods, 111 (1988) 11–16
Elsevier

JIM 04787

A highly specific and sensitive enzyme-immunometric assay for calcitonin gene-related peptide based on enzyme amplification

Rashmi Seth [1], Mone Zaidi [1], James Q. Fuller [2] and Colin H. Self [1]

[1] *Endocrine Unit, Department of Chemical Pathology, Royal Postgraduate Medical School, Du Cane Road, London W12 ONN, U.K., and* [2] *Novo Biolabs Ltd., Downham House, Downhams Lane, Milton Road, Cambridge CB2, U.K.*

(Received 12 January 1988, accepted 22 January 1988)

Calcitonin gene-related peptide (CGRP) is an important member of the peptide family encoded by the calcitonin gene. It has been found to be a potent vasodilator in man and a major circulating gene product (Girgis et al., 1985). The present study reports the development of a sensitive and rapid two-site immunoassay for CGRP based on enzyme amplification (Self, 1985). The assay has been easy to construct, taking advantage of available antisera raised for other purposes. Nevertheless it has been found to be clearly superior to our previous radioimmunoassay in terms of sensitivity, specificity, speed and convenience.

Key words: Calcitonin gene-related peptide; Peptide immunoassay; Amplified immunometric assay; Enzyme amplification

Introduction

Our previous work on the neuropeptide calcitonin gene-related peptide (CGRP) indicated that it was present in normal plasma at a very low concentration and also that the immunoreactivity determined by our previous competitive one-site radioimmunoassy was a mixture of heterogeneous molecular forms including fragments (Zaidi et al., 1986). It was considered that, especially with a competitive immunoassay based on polyclonal antibody directed at a number of different epitopes, such fragments could lead to falsely elevated levels of the peptide. We set out, therefore, to construct an assay for the peptide which was not only highly sensitive but which also would be less open to interference by peptide fragments.

Correspondence to: C.H. Self, Department of Chemical Pathology, Royal Postgraduate Medical School, Du Cane Road, London W12 ONN, U.K.

The two-site immunometric immunoassay is becoming well established in many areas at the expense of competitive one-site approaches. The fact that two-site approaches may provide assays of more sensitivity and specificity has been confirmed with a variety of assay formats and labels. The clear potential advantage of the two-site format to our studies was simply that to be detected, the epitopes against which the two antibodies were directed had to remain joined. We elected therefore to attempt to construct a two-site immunoassay for CGRP. We were not, however, certain at this early stage whether there were sufficiently spatially separated epitopes on this particular small peptide (molecular mass 3786 Da) to support a two-site assay. As a preliminary test of this we decided to attempt to construct a two-site assay with two polyclonal antisera already available, one of which had been raised against the whole molecule but was particularly reactive to the mid-region, while the other had been raised against a C-terminal decapeptide fragment of CGRP.

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In addition we have applied enzyme amplification (Self, 1985) to the assay. This was for two reasons. First, we needed a highly sensitive assay to determine the low normal concentration. Secondly, we were concerned that fragmentation of the peptide might be occurring during the long incubation steps required by the radioimmunoassay. The amplification method allowed us to greatly shorten the length of incubation. As previously described (Johannsson et al., 1985; Self, 1985) the enzyme amplification system used was based on alkaline phosphatase as the enzyme label. This was detected by its ability to dephosphorylate NADP to NAD which was then determined by means of a highly NAD-specific redox cycle (the NAD being first reduced by alcohol dehydrogenase and then the NADH being reoxidised by diaphorase, causing the reduction of an essentially colourless tetrazolium salt to its highly coloured formazan). The high speed of cycling achieved allows rapid build-up of the coloured material even with very small amounts of initial enzyme label. Assays employing this technique have, therefore, been found to be both highly sensitive and fast (Stanley et al., 1985; Lynch et al., 1987).

Materials and methods

Alcohol dehydrogenase (E.C. 1.1.1.1; ADH) and p-iodonitrotetrazolium violet (INT) were obtained from Sigma Chemical Company, Poole, U.K.). Diaphorase (EC 1.6.4.3) was obtained from Boehringer Corporation, London, U.K. Sheep anti-rabbit γ-globulin was obtained from IRE-UK; ^{125}I was obtained from Amersham International PLC, Amersham, Bucks, U.K. Trasylol (aprotinin proteinase inhibitor) was obtained from Bayer, Newbury, Berks., U.K. All other reagents were obtained from BDH, Poole, U.K. and were of the highest grade available.

Antibodies

The antibody, designated CG39, was raised in rabbits against an ovalbumin conjugate of a carboxyl terminal decapeptide analogue of rat CGRP (Zaidi et al., 1986; Zaidi, 1987). It was initially purified on a Bio-Gel P30 column (to be used in RIA) and on a protein A-Sepharose column prior to conjugation with alkaline phosphatase using the maleimide method (Ishikawa et al., 1985). The conjugate was purified by high performance liquid chromatography and used at an initial dilution of 1/100 for EIA. The antibody designed CC2/1, was raised in rabbits against the intact molecule of human CGRP conjugated to ovalbumin and was found to be specific for the mid-region of the molecule (Girgis et al., 1985).

Amplified enzyme linked immunometric assay (AEIA)

Microtitre plates (Nunc, Denmark) were coated by incubating overnight with antibody CC2/1 in sodium bicarbonate buffer (0.020 M, pH 9.6) at a final dilution of 1/2000 (0.10 ml/well). The antibody solution was then removed and the plates washed four times with a solution comprising lactose (5% w/v), casein (0.5% w/v) and Tween-20 (0.02% v/v) in the same buffer. The plates were then dried and stored in a desiccator at room temperature.

Two-fold duplicate dilutions of a standard preparation of synthetic rat CGRP were made in Tris buffer (0.050 M, pH 7.4) containing sodium chloride (0.015 M, pH 7.4) and bovine serum albumin (0.1% w/v). Duplicate dilutions of samples (0.050 ml and 0.10 ml) were dispensed onto the coated plates and incubated either for 2 h or overnight, at room temperature.

Following incubation, the samples were removed by shaking and the plate washed four times with 0.050 M Tris buffer pH 7.4 containing Tween-20 (0.02% v/v). Alkaline phosphatase-labelled CG39 antibody (initial dilution 1/100, 0.10 ml/well) was added and the plate incubated for 2 h at room temperature. The plate was then washed as above and the alkaline phosphatase bound to the plate quantified as follows.

To each well of the microtitre plate, a substrate solution (100 μl of 0.002 M NADP in 0.05 M diethanolamine at pH 9.5) was added and allowed to incubate for 15 min at room temperature. Following this, an amplifier solution (0.2 ml/well of 60 U/ml ADH, 0.1 U/ml diaphorase, 0.00055 M INT and 4% v/v ethanol in 25 mM phosphate buffer at pH 7.0) was mixed into each well and the enzyme reaction allowed to proceed until the colour in the wells containing the highest standard

reached an absorbance unit of 2 at 492 nm (approx. 20 min). The reaction was terminated by the addition of sulphuric acid (0.05 ml/well; 0.2 M) and the absorbances quantified with a Titertek Multiscan MC (Flow Laboratories).

The assay was validated in terms of specificity, sensitivity, precision and recovery. The immunoreactivities of a range of analogues of rat CGRP and a number of structurally related and unrelated peptides were compared to that of rCGRP. Sensitivity was calculated as being the lowest concentration of the peptide that could be confidently distinguished from zero (blank $\pm$ 2.0 SD). Dose-error relationships (precision profiles) were constructed from duplicate dilutions of standards and samples. The intra- and inter-assay variations were calculated from two pools (6.25 and 12.5 fmol/tube synthetic rCGRP) used in different assays.

Radioimmunoassay

A radioimmunoassay using antibody CG39 has already been described (Zaidi et al., 1986). An HPLC-purified ^{125}I-iodotyrosyl-rCGRP (28–37) (specific activity 1800 Ci/mmol, 95% pure) was prepared in our laboratory. The inter- and intra-assay variation has previously been shown to be $\leqslant 10\%$ and the assay has been found to have a working range of 2–15 fmol/tube (coefficient of variation 15%) (Zaidi, 1987).

Animal experiments and extraction

Following an overnight fast, male Wistar rats (148 $\pm$ 3.9 g, $n = 15$) were anaesthetised with light ether and exsanguinated via the dorsal aorta. Blood was collected into heparinised syringes, separated within 30 min of collection and stored at $-20\,^{\circ}$C.

The extraction process, modified from Bennett's protocol (Bennett et al., 1978), was similar to that described earlier (Zaidi et al., 1986). Plasma was acidified with a medium comprising formic acid (5% v/v), trifluoroacetic acid (TFA, 15% v/v), hydrochloric acid (1 M), mercaptoethanol (0.5 M) and sodium chloride (1% w/v). Following incubation with the above medium for 1 h, plasma was centrifuged at $500 \times g$ and 4°C for 15 min, and the supernatant passed through prewetted Sep-pak C18 cartridges (Water Associates, Milford, MA) three times. The peptide was finally eluted in a solution containing methanol (80% v/v) and aqueous TFA (0.5% v/v). The eluate was dried in a vacuum oven and reconstituted in phosphate buffer (0.05 M, pH 7.4) containing aprotinin proteinase inhibitor (40 IU/ml Trasylol; Bayer, Newbury, Berks) on the day of assay. The mean recovery of synthetic rCGRP added to charcoal stripped plasma was 75% $\pm$ 3.6% (by RIA) and the variation between extractions was 15%.

TABLE I

SENSITIVITY OF THE AEIA COMPARED WITH RIA CONDUCTED WITH VARIOUS INCUBATION PERIODS

The assays were conducted as described. For the RIAs with extended incubation periods (2+1) days refers to an incubation of 2 days of the CGRP with specific antibody followed by an incubation of 1 day with labelled CGRP and similarly (3+2) days refers to 3 days' incubation with antibody followed by 2 days with labelled CGRP.

	Sensitivity			
	2 h	Overnight	2+1 days	3+2 days
AEIA	2.5 fmol/well	0.25 fmol/well	–	–
RIA	–	4 fmol/tube	0.5 fmol/tube	0.25 fmol/tube

Results

Sensitivity

Table I compares the sensitivity of the AEIA with that of RIA with various incubation protocols. The amplified assay was found to be at least 10 times more sensitive than the corresponding RIA with overnight incubation. When the AEIA incubation protocol was changed from overnight to a few hours, the sensitivity of the system was somewhat reduced but still remained clearly superior to the RIA.

Precision

The mean intra- and inter-assay variability was 6 and 10% respectively for the AEIA. Dose-error relationships (precision profiles) were constructed from duplicate dilutions of standards and samples (Fig. 2). Between 5 and 20 fmol/tube or well the coefficients of variation were less than 10% and 15% for the AEIA and RIA respectively.

TABLE II

CROSS-REACTIVITY OF VARIOUS CGRP ANALOGUES WITH CGRP WHEN MEASURED WITH THE RIA AND THE AEIA

CGRP homologue/analogue	% Cross-reactivity	
	AEIA	RIA
rCGRP	100	100
αhCGRP	70	77
βhCGRP	70	166
α1–11 hCGRP	⩽ 0.1	⩽ 0.1
α1–16 hCGRP	⩽ 0.1	⩽ 0.1
α1–18 hCGRP	⩽ 0.1	⩽ 0.1
α17–37 hCGRP	60	40
α19–37 hCGRP	⩽ 0.1	11

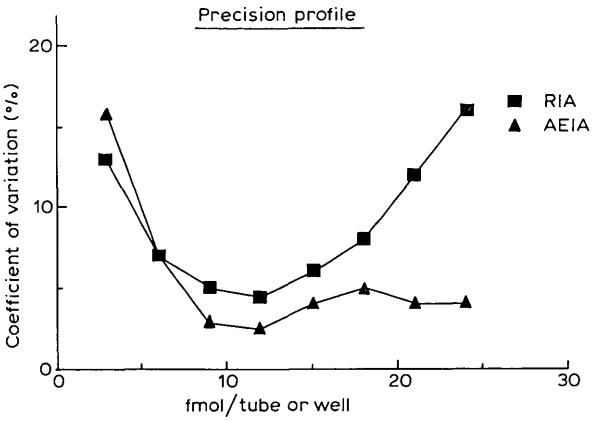


Fig. 2. Within-assay precision profile from pooled values of immunoreactive CGRP, in extracts of rat plasma obtained using a 3 + 2 day incubation protocol for RIA and an overnight protocol for AEIA.

Specificity

The two-site AEIA was found to be more specific for CGRP than was the one-site RIA with respect to the full range of CGRP peptides tested as shown in Table II. Of importance was the much lower cross-reactivity of α19–37 hCGRP in the AEIA.

Recovery

Typical standard curves for rCGRP were obtained, as shown in Fig. 1. Also shown in this figure are the results obtained as a consequence of diluting extracted plasma samples with the assay buffer for both assays. Both the RIA and the AEIA exhibited parallel dilution characteristics. However, when the samples were assayed by the two methods, only a weak correlation was found ($r = 0.61$, $P \leqslant 0.05$) between values obtained from

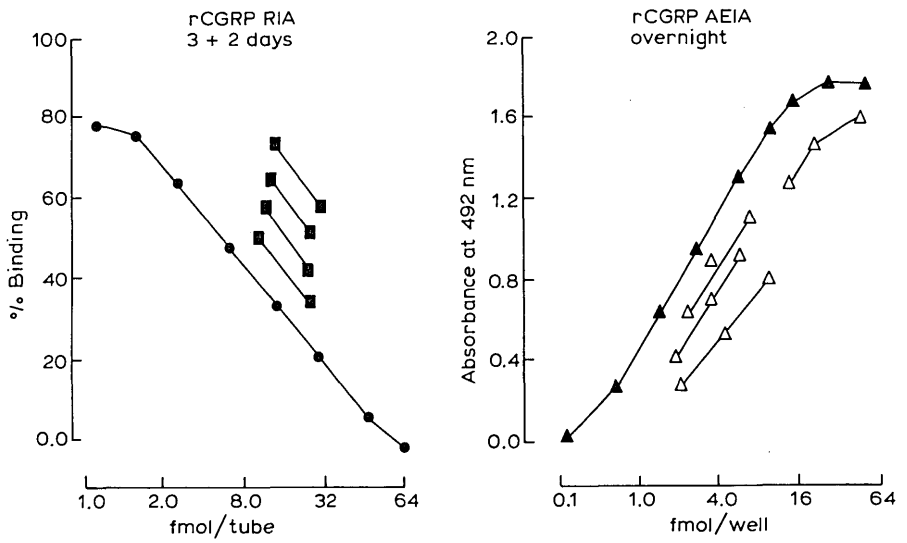


Fig. 1. Comparison of typical standard curves obtained for AEIA and RIA. Parallel dilution of samples was obtained with both methods.

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Discussion

The enzyme amplified two-site assay we have developed for CGRP is fast, highly sensitive and specific. With overnight incubation the sensitivity is 250 amol per tube. When such high sensitivity is not required it is possible to make the assay much faster by employing only a 2 h incubation and still obtain the sensitivity and precision given by our previous RIA with an overnight incubation. Such an assay is of particular use in monitoring CGRP levels during, for example, purification procedures.

The two-site assay appeared to be more specific, especially with regard to fragments, than our comparable RIA. This finding was expected in view of the requirement for two antibodies to bind the analyte in the two-site assay for the presence of material to be detected. The two-site nature of the assay also allowed us to reduce interfering background. This was achieved by adopting a sequential protocol. The sample was incubated with the plate-bound antibody first. Unbound, possibly interfering, material was washed off the plate and only then was the labelled antibody added and incubated with the captured analyte. The weak correlation between the AEIA and RIA was not unexpected. Neither was the finding that mean plasma levels of CGRP were apparently higher when measured by the RIA.

The overall high performance of the AEIA was somewhat surprising. This was because it had been constructed with unpurified polyclonal antibodies, including one which has been raised against the whole molecule, which were tested because they were to hand. It is likely that the assay could be further improved. The antibodies, especially that raised against the whole molecule could be purified as in the study carried out on the similarly small peptide ACTH (Hodgkinson et al., 1984). Also, now that we have demonstrated the feasibility of a two-site assay for CGRP, it would be worth investing the effort required to raise monoclonal antibodies. However, the simple approach we have taken serves to underline the ease with which enzyme amplification can be usefully applied to peptide determination using available antibodies.

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Journal of Immunological Methods, 111 (1988) 11–16
Elsevier

JIM 04787

A highly specific and sensitive enzyme-immunometric assay for calcitonin gene-related peptide based on enzyme amplification

Rashmi Seth [1], Mone Zaidi [1], James Q. Fuller [2] and Colin H. Self [1]

[1] *Endocrine Unit, Department of Chemical Pathology, Royal Postgraduate Medical School, Du Cane Road, London W12 ONN, U.K., and* [2] *Novo Biolabs Ltd., Downham House, Downhams Lane, Milton Road, Cambridge CB2, U.K.*

(Received 12 January 1988, accepted 22 January 1988)

Calcitonin gene-related peptide (CGRP) is an important member of the peptide family encoded by the calcitonin gene. It has been found to be a potent vasodilator in man and a major circulating gene product (Girgis et al., 1985). The present study reports the development of a sensitive and rapid two-site immunoassay for CGRP based on enzyme amplification (Self, 1985). The assay has been easy to construct, taking advantage of available antisera raised for other purposes. Nevertheless it has been found to be clearly superior to our previous radioimmunoassay in terms of sensitivity, specificity, speed and convenience.

Key words: Calcitonin gene-related peptide; Peptide immunoassay; Amplified immunometric assay; Enzyme amplification

Introduction

Our previous work on the neuropeptide calcitonin gene-related peptide (CGRP) indicated that it was present in normal plasma at a very low concentration and also that the immunoreactivity determined by our previous competitive one-site radioimmunoassy was a mixture of heterogeneous molecular forms including fragments (Zaidi et al., 1986). It was considered that, especially with a competitive immunoassay based on polyclonal antibody directed at a number of different epitopes, such fragments could lead to falsely elevated levels of the peptide. We set out, therefore, to construct an assay for the peptide which was not only highly sensitive but which also would be less open to interference by peptide fragments.

Correspondence to: C.H. Self, Department of Chemical Pathology, Royal Postgraduate Medical School, Du Cane Road, London W12 ONN, U.K.

The two-site immunometric immunoassay is becoming well established in many areas at the expense of competitive one-site approaches. The fact that two-site approaches may provide assays of more sensitivity and specificity has been confirmed with a variety of assay formats and labels. The clear potential advantage of the two-site format to our studies was simply that to be detected, the epitopes against which the two antibodies were directed had to remain joined. We elected therefore to attempt to construct a two-site immunoassay for CGRP. We were not, however, certain at this early stage whether there were sufficiently spatially separated epitopes on this particular small peptide (molecular mass 3786 Da) to support a two-site assay. As a preliminary test of this we decided to attempt to construct a two-site assay with two polyclonal antisera already available, one of which had been raised against the whole molecule but was particularly reactive to the mid-region, while the other had been raised against a C-terminal decapeptide fragment of CGRP.

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In addition we have applied enzyme amplification (Self, 1985) to the assay. This was for two reasons. First, we needed a highly sensitive assay to determine the low normal concentration. Secondly, we were concerned that fragmentation of the peptide might be occurring during the long incubation steps required by the radioimmunoassay. The amplification method allowed us to greatly shorten the length of incubation. As previously described (Johannsson et al., 1985; Self, 1985) the enzyme amplification system used was based on alkaline phosphatase as the enzyme label. This was detected by its ability to dephosphorylate NADP to NAD which was then determined by means of a highly NAD-specific redox cycle (the NAD being first reduced by alcohol dehydrogenase and then the NADH being reoxidised by diaphorase, causing the reduction of an essentially colourless tetrazolium salt to its highly coloured formazan). The high speed of cycling achieved allows rapid build-up of the coloured material even with very small amounts of initial enzyme label. Assays employing this technique have, therefore, been found to be both highly sensitive and fast (Stanley et al., 1985; Lynch et al., 1987).

Materials and methods

Alcohol dehydrogenase (E.C. 1.1.1.1; ADH) and p-iodonitrotetrazolium violet (INT) were obtained from Sigma Chemical Company, Poole, U.K.). Diaphorase (EC 1.6.4.3) was obtained from Boehringer Corporation, London, U.K. Sheep anti-rabbit γ-globulin was obtained from IRE-UK; ^{125}I was obtained from Amersham International PLC, Amersham, Bucks, U.K. Trasylol (aprotinin proteinase inhibitor) was obtained from Bayer, Newbury, Berks., U.K. All other reagents were obtained from BDH, Poole, U.K. and were of the highest grade available.

Antibodies

The antibody, designated CG39, was raised in rabbits against an ovalbumin conjugate of a carboxyl terminal decapeptide analogue of rat CGRP (Zaidi et al., 1986; Zaidi, 1987). It was initially purified on a Bio-Gel P30 column (to be used in RIA) and on a protein A-Sepharose column prior to conjugation with alkaline phosphatase using the maleimide method (Ishikawa et al., 1985). The conjugate was purified by high performance liquid chromatography and used at an initial dilution of 1/100 for EIA. The antibody designed CC2/1, was raised in rabbits against the intact molecule of human CGRP conjugated to ovalbumin and was found to be specific for the mid-region of the molecule (Girgis et al., 1985).

Amplified enzyme linked immunometric assay (AEIA)

Microtitre plates (Nunc, Denmark) were coated by incubating overnight with antibody CC2/1 in sodium bicarbonate buffer (0.020 M, pH 9.6) at a final dilution of 1/2000 (0.10 ml/well). The antibody solution was then removed and the plates washed four times with a solution comprising lactose (5% w/v), casein (0.5% w/v) and Tween-20 (0.02% v/v) in the same buffer. The plates were then dried and stored in a desiccator at room temperature.

Two-fold duplicate dilutions of a standard preparation of synthetic rat CGRP were made in Tris buffer (0.050 M, pH 7.4) containing sodium chloride (0.015 M, pH 7.4) and bovine serum albumin (0.1% w/v). Duplicate dilutions of samples (0.050 ml and 0.10 ml) were dispensed onto the coated plates and incubated either for 2 h or overnight, at room temperature.

Following incubation, the samples were removed by shaking and the plate washed four times with 0.050 M Tris buffer pH 7.4 containing Tween-20 (0.02% v/v). Alkaline phosphatase-labelled CG39 antibody (initial dilution 1/100, 0.10 ml/well) was added and the plate incubated for 2 h at room temperature. The plate was then washed as above and the alkaline phosphatase bound to the plate quantified as follows.

To each well of the microtitre plate, a substrate solution (100 μl of 0.002 M NADP in 0.05 M diethanolamine at pH 9.5) was added and allowed to incubate for 15 min at room temperature. Following this, an amplifier solution (0.2 ml/well of 60 U/ml ADH, 0.1 U/ml diaphorase, 0.00055 M INT and 4% v/v ethanol in 25 mM phosphate buffer at pH 7.0) was mixed into each well and the enzyme reaction allowed to proceed until the colour in the wells containing the highest standard

reached an absorbance unit of 2 at 492 nm (approx. 20 min). The reaction was terminated by the addition of sulphuric acid (0.05 ml/well; 0.2 M) and the absorbances quantified with a Titertek Multiscan MC (Flow Laboratories).

The assay was validated in terms of specificity, sensitivity, precision and recovery. The immunoreactivities of a range of analogues of rat CGRP and a number of structurally related and unrelated peptides were compared to that of rCGRP. Sensitivity was calculated as being the lowest concentration of the peptide that could be confidently distinguished from zero (blank $\pm$ 2.0 SD). Dose-error relationships (precision profiles) were constructed from duplicate dilutions of standards and samples. The intra- and inter-assay variations were calculated from two pools (6.25 and 12.5 fmol/tube synthetic rCGRP) used in different assays.

Radioimmunoassay

A radioimmunoassay using antibody CG39 has already been described (Zaidi et al., 1986). An HPLC-purified ^{125}I-iodotyrosyl-rCGRP (28–37) (specific activity 1800 Ci/mmol, 95% pure) was prepared in our laboratory. The inter- and intra-assay variation has previously been shown to be $\leqslant 10\%$ and the assay has been found to have a working range of 2–15 fmol/tube (coefficient of variation 15%) (Zaidi, 1987).

Animal experiments and extraction

Following an overnight fast, male Wistar rats (148 $\pm$ 3.9 g, $n = 15$) were anaesthetised with light ether and exsanguinated via the dorsal aorta. Blood was collected into heparinised syringes, separated within 30 min of collection and stored at $-20\,^{\circ}$C.

The extraction process, modified from Bennett's protocol (Bennett et al., 1978), was similar to that described earlier (Zaidi et al., 1986). Plasma was acidified with a medium comprising formic acid (5% v/v), trifluoroacetic acid (TFA, 15% v/v), hydrochloric acid (1 M), mercaptoethanol (0.5 M) and sodium chloride (1% w/v). Following incubation with the above medium for 1 h, plasma was centrifuged at $500 \times g$ and 4 °C for 15 min, and the supernatant passed through prewetted Sep-pak C18 cartridges (Water Associates, Milford, MA) three times. The peptide was finally eluted in a solution containing methanol (80% v/v) and aqueous TFA (0.5% v/v). The eluate was dried in a vacuum oven and reconstituted in phosphate buffer (0.05 M, pH 7.4) containing aprotinin proteinase inhibitor (40 IU/ml Trasylol; Bayer, Newbury, Berks) on the day of assay. The mean recovery of synthetic rCGRP added to charcoal stripped plasma was 75% $\pm$ 3.6% (by RIA) and the variation between extractions was 15%.

TABLE I

SENSITIVITY OF THE AEIA COMPARED WITH RIA CONDUCTED WITH VARIOUS INCUBATION PERIODS

The assays were conducted as described. For the RIAs with extended incubation periods (2+1) days refers to an incubation of 2 days of the CGRP with specific antibody followed by an incubation of 1 day with labelled CGRP and similarly (3+2) days refers to 3 days' incubation with antibody followed by 2 days with labelled CGRP.

	Sensitivity			
	2 h	Overnight	2+1 days	3+2 days
AEIA	2.5 fmol/well	0.25 fmol/well	–	–
RIA	–	4 fmol/tube	0.5 fmol/tube	0.25 fmol/tube

Results

Sensitivity

Table I compares the sensitivity of the AEIA with that of RIA with various incubation protocols. The amplified assay was found to be at least 10 times more sensitive than the corresponding RIA with overnight incubation. When the AEIA incubation protocol was changed from overnight to a few hours, the sensitivity of the system was somewhat reduced but still remained clearly superior to the RIA.

Precision

The mean intra- and inter-assay variability was 6 and 10% respectively for the AEIA. Dose-error relationships (precision profiles) were constructed from duplicate dilutions of standards and samples (Fig. 2). Between 5 and 20 fmol/tube or well the coefficients of variation were less than 10% and 15% for the AEIA and RIA respectively.

TABLE II

CROSS-REACTIVITY OF VARIOUS CGRP ANALOGUES WITH CGRP WHEN MEASURED WITH THE RIA AND THE AEIA

CGRP homologue/analogue	% Cross-reactivity	
	AEIA	RIA
rCGRP	100	100
αhCGRP	70	77
βhCGRP	70	166
α1–11 hCGRP	⩽ 0.1	⩽ 0.1
α1–16 hCGRP	⩽ 0.1	⩽ 0.1
α1–18 hCGRP	⩽ 0.1	⩽ 0.1
α17–37 hCGRP	60	40
α19–37 hCGRP	⩽ 0.1	11

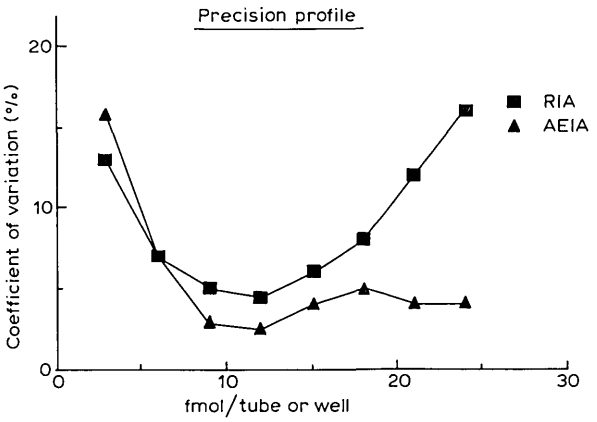


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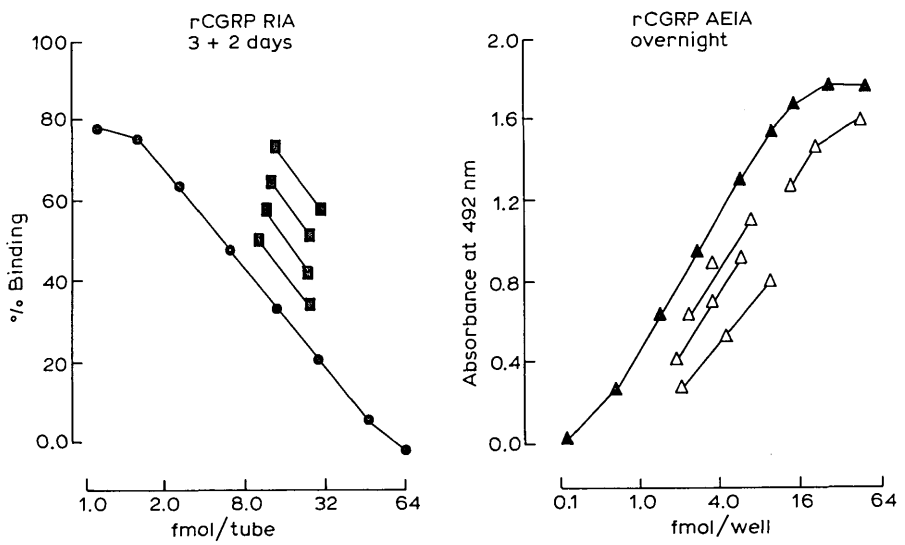


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