Title: NMR-based metabonomic analysis of normal rat urine and faeces in response to (±)-venlafaxine treatment

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Abbreviations: GMBLKICA, gut-microbiome-brain-liver-kidney-immune-cardiovascular system axis, CV-ANOVA, ANOVA of the cross-validated residuals, PLS BP, PLS-based batch processing.

Highlights

- (±)-Venlafaxine decreases urinary levels of co-metabolites and osmolytes.
- (±)-Venlafaxine decreases faecal levels of co-metabolites.
- The decreased urinary level of hippurate is a dose-dependent effect.

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Abstract

(±)-Venlafaxine, a bicyclic antidepressant of the serotonin-norepinephrine reuptake inhibitor (SNRI) class, is prescribed for the treatment of depression and anxiety disorders. As is the case with other antidepressants, its precise mechanisms of action are still unknown. Pharmacometabonomic approaches allow for the detection of diverse metabolites, unlike classic methods for analysing drug interaction based on single metabolites and linear pathways. This provides a global view of the state of homeostasis during treatment and an insight into the mechanisms of action of a drug. Accordingly, the final outcome of treatment is characterised by the network of reactome pathways derived from the on-target and off-target effects of the drug. Regarding antidepressants, the drug network may be located in the gut-microbiome-brain-liver-kidney-immune-cardiovascular system axis (GMBLKICA), implying that neurotransmitters participate as signalling molecules in bidirectional communication. If their bioavailability is increased, this communication and the state of homeostasis may be disrupted. With a pharmacometabonomic approach using NMR in combination with different chemometric methods, a determination was made of subtle changes in the metabolic profile (metabotype) of urine and faeces in normal Wistar rats following a single administration of pharmacological doses of (±)-venlafaxine hydrochloride. Based on the drug-response metabolotypes observed, (±)-venlafaxine had effects on gut microbial co-metabolites and osmolytes. Hence, it can be hypothesized that bidirectional communication in the multiorgan axis was perturbed by this drug, and very likely by its active metabolite, (±)-desvenlafaxine. This disrupted signalling could be related not only to therapeutic and adverse effects, but also to the lag period in treatment response.

1. Introduction

Depression is a common illness that affects 350 million people worldwide (WHO, www.who.int). Since this disorder is varied and complex, its etiology and pathology remain unknown, as do the mechanisms of action of the antidepressants currently used. Among the various hypotheses about depression, the most relevant is the monoamine hypothesis [1]. Since this disorder is multifactorial, its study requires a multidisciplinary approach that can only be carried out with the use of recent technological advances such as genomics, proteomics and metabonomics. Data from these methodologies can be integrated to generate suitable hypotheses about the etiology of diseases and the mechanisms of action of a drug.

NMR-based metabonomics is a rapid, non-destructive and non-invasive analytical method that allows for the detection of a wide range of structurally diverse metabolites in a single experiment, provides highly reproducible results [2], and reveals the time-related metabolic effects of drugs, drug carrier vehicles and toxins [3-7]. Pharmacometabonomics is a new field that uses metabonomics tools to provide information regarding the mechanisms of action of drugs, which in turn can lead to a better understanding of drug efficacy and side effects, as well as the variation in response to treatment [8]. Pharmacometabonomics is not limited by prior understanding or hypotheses. Indeed, it provides detailed biochemical fingerprints of pharmaceutical effects on metabolism, which can lead to the generation of new hypotheses regarding the way in which drugs produce their effects. Its application can be useful in preclinical and clinical trials to select lead compounds at early stages of testing, and in pharmacovigilance studies to ensure the safe use of medications [9,10].

The pharmacometabonomic concept is also integral to the understanding of the combinatorial metabolic interactions between the gut microbiome and the host, as well as the consequences of the same in terms of the impact on disease and therapy. This co-metabolism can contribute to PK/PD profiles of a drug and the variations in response to treatment [11]. The metabonome represents the sum of the interactions of all the individual metabolomes and their products within a complex organism. This includes extended genomic, symbiotic, parasitic, environmental and co-metabolic interactions. Since urine, faeces and plasma are fluids that carry metabolic signatures resulting from such interactions [12], it is useful to analyse more than one biofluid to obtain appropriate information and generate hypotheses about the process of pathogenesis or response to treatment.

Because the gut microbiome contributes to the metabolome [8], a metabonomic approach analyses the human body as a supraorganism, in which the host and the microbiome are in constant bidirectional communication to maintain homeostasis inside the so-called gut-microbiome-brain-liver-immune system axis [13]. The gut microbiome is a virtual organ with endocrine capacity, able to monitor the host and of course vice versa through neuroendocrine-bacterial interactions [14].

Recent studies have suggested that by these interactions the microbiome is involved in neurological functions, leading to effects on mood and behaviour by different pathways [15-22]. If this is indeed the case, the precise mechanisms are as yet unclear. However, it is known that modulation of mood and behaviour is related to the serotonergic and adrenergic pathways, which are involved in bidirectional communication between the brain and the gut via enterochromaffin cells (ECs) as well as the gut microbiome, enteric nervous system (ENS), autonomic nervous system (ANS), hypothalamus-pituitary-adrenal (HPA) axis and central nervous system (CNS). It is through the HPA axis that the gastrointestinal tract (GI) and its microbiome are associated with neurological function and vice versa in normal physiology and disease. Alterations in these continuous interactions are believed to be involved in pathogenesis affecting the gut-microbiome-brain axis, leading to clinical manifestations of behavioural disorders and physical maladies such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [23-27]. Also, disruptions in these interactions have been implicated in the pathophysiology of autism spectrum disorders (ASDs) [28]. In animal models such disruptions are associated with depression and alterations in the gut microbiome [17].

Alteration of the gut microbiome involved in the modification of behaviour can be detected by analysing the metabolic phenotypes (metabotypes) generated from biofluids. For instance, faecal extracts of IBD patients have been characterized by reduced levels of short-chain fatty acids (SCFAs) and monoamines derived from gut microbial metabolism [29], and this GI disorder is comorbid with depression [18,30]. In another report, the urinary phenotype of autistic children showed fluctuations of co-metabolites, associating this brain disorder with increased levels of *p*-cresol [31]. Moreover, in animal models with antibiotics treatment, which has been linked to the modification of behaviour [18,26], levels of co-metabolites have been affected [4,32]. In this regard, the reactome of a drug includes on-target and off-target activities whose pathways form an interaction network that produces pharmacological and toxicological effects [33-37], this being characterised by the drug-response phenotype [3,10].

In the present work, an untargeted NMR-based pharmacometabonomic analysis was performed to understand the response in normal rats to (±)-venlafaxine, a serotonin and norepinephrine reuptake inhibitor (SNRI) that was administered at pharmacological doses. This approach suggests that the present treatment affects the gut microbiome and kidney, evidenced by changes in urinary and faecal metabolic profiles.

2. Materials and methods

2.1. Chemicals

(±)-Venlafaxine was extracted from 75 mg Vextor® (Torrent Pharmaceuticals Ltd., India). The content of 60 extended-release capsules of venlafaxine hydrochloride was pulverized in a mortar and then added to 7% HCl mixed with 200 mL CH₂Cl₂ (2:1), after which the mixture was filtered. The aqueous layer was extracted from the filtrate and the organic layer was washed with 7% HCl (3 x 50 mL). A mixture of 10% NaOH and 200 mL CH₂Cl₂ (2:1) was added to the combined aqueous layers and then brine was poured into the mixture to produce the rupture of the emulsion that formed. The aqueous layer was washed with CH₂Cl₂ (3 x 60 mL), dried (anh. Na₂SO₄), and evaporated *in vacuo* to give a white solid (venlafaxine free base, 62 %). The solid was dissolved in EtOAc and treated with 10 % HCl in isopropanol. Afterwards the mixture was dried (anh. Na₂SO₄) and concentrated *in vacuo* to afford a white solid (venlafaxine hydrochloride, 88.3 %). The product was recrystallized from methanol/ethyl acetate (1:2) [38].

(±)-O-Desmethylvenlafaxine ((±)-desvenlafaxine) was extracted from Pristiq® 50 mg (Wyeth, Puerto Rico). Ten extended-release tablets of (±)-desvenlafaxine succinate monohydrate were pulverized and suspended in a mixture of 7% HCl and 200 mL EtOAc (2:1) and then filtered. The aqueous layer was extracted and the organic layer was washed with 7% HCl (3 x 50 mL). The combined aqueous layers were neutralized with 10% NaOH, and then 100 mL EtOAc was added. Brine was added to produce the rupture of the emulsion that formed. The aqueous layer was washed with EtOAc (3 x 60 mL), dried (anh. Na₂SO₄), and evaporated *in vacuo* to give a white solid (O-desmethylvenlafaxine free base, 65.2 %). The product was recrystallized from methanol/ethyl acetate (1:2).

1.0 g of (±)-N,N-Didesmethylvenlafaxine hydrochloride (Sinbiotik, Mexico) was dissolved in 70 mL DCM and treated with 2.0 % w/v NaOH (100 mL). The aqueous layer was washed with DCM (3 x 30 mL). The combined organic layers were dried (anh. Na₂SO₄) and concentrated *in vacuo* to give a transparent oil (N,N-didesmethylvenlafaxine free base, 60%).

For all these standards, 1D ^1H , ^{13}C and 2D NMR spectra were acquired in CDCl_3 to confirm their purity, chemical shifts and multiplicity.

2.2. Animal handling and sample collection

Fifty-four adult female Wistar rats (body weight, 270 ± 10 g) were kept in polycarbonate cages (Allentown Inc., Allentown, NJ, US) and allowed to acclimatize themselves for seven days under controlled environmental conditions (temperature, 22-24 °C; relative humidity, 50-55%; day/night cycle, 12-12 h, lights on at 7 a.m.), with access to standard rodent diet (PMI Nutrition International, LLC. rodent laboratory chow 5001, Brentwood, MO) and water *ad libitum*. All experimental procedures were carried out in accordance with the guidelines provided by the laws and regulations of Mexico in the Seventh Title of the Regulations of the General Law of Health Regarding Health Research and the Mexican Official Standard (NOM-082-ZOO-1999) with respect to the care and use of laboratory animals.

After the one-week adjustment period, animals were randomly allocated to three groups ($n = 18$) and administered p.o. a single dose of either vehicle (5% Tween-80 w/v) or venlafaxine hydrochloride combined with vehicle (at 22 or 112 mg/kg of b.w. of venlafaxine free base, dose volume 4 mL/kg b.w.). Before sample collection, the bladders of animals were emptied by gentle pressure on the lower part of the abdomen to induce urination. Then, the animals were housed in individual metabolic cages designed to avoid contamination of the urine and separate it from stool into collection tubes (3M12D100/3700M071, Tecniplast, Buguggiate, Va, Italy). The animals were food deprived in accordance with a previously reported procedure [39]. Briefly, the rats were fasted for 16 h prior to the drug or vehicle administration and food was returned 4 h after dosing. Animals has access to water *ad libitum* throughout the study. Urine samples were collected at intervals of 0 to 6 h, 6 to 12 h and 12 to 24 h. The collection period of stool samples was 0 to 24 h. All samples were stored at -80°C to await analysis.

2.3. Sample Preparation for NMR Spectroscopy

The urine samples were thawed, vortexed, and allowed to stand for 10 min at room temperature prior to mixing 440 μL with 220 μL phosphate buffer [0.2 M, pH 7.4, containing 0.1 % (w/v) of sodium azide (Sigma Aldrich)]. D_2O (99.9% in D , Sigma Aldrich) was used to provide a field frequency lock and 3-trimethylsilyl-[2,2,3,3- $^2\text{H}_4$]-propionic acid sodium salt (TSP, Sigma Aldrich) as a chemical shift reference (^1H , δ 0.0). The urine-buffer mixtures were centrifuged at 15,600 g for 10 min at room temperature. The supernatants (540 μL)

were transferred to 5 mm NMR tubes and 60 μ L of a TSP/D₂O solution was added to give a final TSP concentration of 1 mM [11].

The faecal samples were homogenized with 0.2 M phosphate buffer (as aforementioned, 5 mL of buffer per gram of stool). Homogenates were subjected to 10 cycles of sonication-vortex-break (10 s per stage). Following the centrifugation (15,600 *g* at room temperature) for 10 min, the supernatants (540 μ L) were pipetted into 5 mm NMR tubes and then 60 μ L of a TSP/D₂O solution was added (as aforementioned) for NMR analysis [40].

2.4. NMR spectroscopy

NMR spectra were acquired on a Varian NMR system 500 spectrometer operating at 499.8 MHz (¹H frequency) and at 298 K. The one-dimensional ¹H NMR spectra of urine and stool water were acquired using a standard one-dimensional pulse sequence NOESYPR (recycle delay-90°-t₁-90°-t_m-90°-acquisition) where t₁ represented the first increment in the NOESY experiment and was set to 3 μ s. Water presaturation was used during both the recycle delay (1 s) and mixing time (t_m, 100 ms), providing an acquisition time of 4 s. For each sample, 128 transients (32 dummy scans) were collected into 64k data points over a 20 ppm spectral width. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz, and data were zero-filled to 64k data points prior to Fourier transformation (FT) [2].

Two-dimensional NMR analysis was performed on selected samples (of both urine and stool water) for identification of endogenous, drug and vehicle metabolites. For the 2D ¹H J-resolved spectroscopy (JRES), spectra were acquired using a HOMO2DJ pulse sequence where 64 transients (32 dummy scans) per increment and 128 increments were collected with an acquisition time of 0.625 s, and a relaxation delay of 1.2 s. The spectral widths in F1 and F2 were 64 Hz and 7 KHz, respectively. Spectra were processed using a SINEBELL function in both dimensions to result in a resolution of ~4.3k (F2) and 128 (F1). The peaks were subsequently tilted by 45° and symmetrised. Two dimensional ¹H-¹H total correlation spectroscopy (TOCSY) spectra were performed via zTOCSY pulse sequence using the DIPSI2 spin-lock scheme and a spectral width of 7 KHz in both dimensions and a mixing time of 80 ms; 32 transients per increment (32 dummy scans) were collected into 256 increments with a relaxation delay of 1.2 s and acquisition time of 0.15 s. Spectra were processed into a resolution of ~1k (F2) and 256 (F1) using a GAUSSIAN function in both dimensions. 2D ¹H-¹³C heteronuclear multiple quantum correlation (HMQC) spectra were collected using the ASAPHMQC pulse sequence. Experimental parameters were set

as follows: 256 increments, 64 transients, 8 dummy scans, a spectral width of 7 KHz and 29 KHz for the ^1H and ^{13}C axes, respectively, with a relaxation delay of 0.3 s and acquisition time of 64 ms. Spectra were processed into a resolution of 435 (F2) and 256 (F1) using a GAUSSIAN function in both dimensions. The 2D ^1H - ^1H correlation spectroscopy (COSY) was performed only on the urine compartment. The gCOSY pulse sequence was used with 16 transients (8 dummy scans) per increment and 256 increments collected with an acquisition time of 0.15 s and a relaxation delay of 1.2 s. The spectral width was 7 KHz in both dimensions. The spectrum were processed into a resolution of $\sim 1\text{k}$ (F2) and 256 (F1) using a SQSINEBELL function in both dimensions. For all 2D spectra water presaturation was performed during the relaxation delay.

2.5. NMR spectral data processing

The 1D ^1H NMR spectra were phased, baseline corrected and referenced to TSP at δ 0.0 by manually using Agilent VnmrJ 4.2. Full resolution 1D ^1H NMR spectra ($\sim 22\text{k}$ data points) were imported into MatLab (R2014a, The MathWorks Inc., Natick, MA). For urine spectra, the spectral region δ 4.12-6.47 containing residual water and urea resonances was removed prior to normalisation by the probabilistic quotient method [41]. In order to remove the drug-related peaks and recover the endogenous metabolic data, the STOCSY-E algorithm was run on the venlafaxine data using the driver peak at δ 2.828, which corresponds to the non-overlapped resonance from the singlet $\text{N}-(\text{CH}_3)_2$ of ($\pm$)-venlafaxine-related compounds [42]. The urinary spectral regions containing the ($\pm$)-venlafaxine xenometabolome resonances (δ 1.09–1.10, 1.11–1.18, 1.23–1.61, 1.66–1.87, 2.65–2.67, 2.79–2.86, 3.06–3.10; 3.54–3.57; 3.85–3.87; 6.92–6.97; 7.02–7.08; 7.16–7.19 and 7.33–7.41) were separated from the remaining spectral regions to form a xenobiotic spectral data set [3]. The fraction of the dose remaining (FDR) was calculated and correlated with the drug metabotype and time in order to be able to observe any relationship with the perturbation of the endogenous metabotype.

$$\text{FDR} = (1/2)^n, \text{ where } n = t/t_{1/2} \text{ [43]}$$

Where $t = \{6, 12, 24\}$, and $t_{1/2}$ = the half-life of ($\pm$)-venlafaxine hydrochloride in rat = 1.2h [44].

For faecal water spectra, the region containing the water resonance (δ 4.07-5.75) was removed and normalised using the total area method. The vehicle-derived resonances were removed via the STOCSY-E algorithm with the driver peak at δ 3.712, which corresponds to the sorbitan resonances [42]. It is known that tween 80 is hydrolysed by

lipases to release the polyoxyethylene sorbitan moiety, which is poorly absorbed in the rat gastrointestinal tract and excreted unchanged in faeces [4]. For both compartments the region corresponding to TSP (δ -0.20–0.50) was removed.

2.6. Identification of metabolites

The structural identification of metabolites in urine and stool water was achieved by 2D NMR experiments and statistical total correlation spectroscopy (STOCSY) [45]. Literature [46-48] or databases, such as the Human Metabolome Data Base (HMDB; <http://www.hmdb.ca/>) or the Biological Magnetic Resonance Data Bank (BMRB; <http://www.bmrwisc.edu>), along with Chenomx NMR Suite 8.0 (Chenomx Inc., Edmonton, Alberta, Canada), were used for confirmation of assignments.

To complement the information obtained from 1D and 2D NMR spectra of the venlafaxine-derived standards in CDCl_3 and to confirm the signals of the drug metabolome in urine, millimolar solutions were prepared in phosphate buffer and analysed by 1D ^1H NMR experiment using the aforementioned procedure. Additionally, 2D NMR spectra were acquired from two urine samples of the high dose group, one at each of the first two points in time. On these spectra, the venlafaxine xenometabolome resonances were predominant.

2.7. Multivariate data analysis

2.7.1. Stationary model

Multivariate data analysis was performed with SIMCA software (version 13.0 Umetrics, Sweden). Principal component analysis (PCA), partial least squares (PLS) and orthogonal projection to latent structure (OPLS) were constructed using Pareto-scaled NMR data, considering each time separately. PLS and OPLS were validated by both a 7-fold cross-validation and ANOVA of the cross-validated residuals (CV-ANOVA) methods. The PLS model was also validated via the permutation test (200 permutations) [49,50]. The PLS and OPLS models were constructed to investigate the statistical relationship between the NMR spectral data (X) and the dose ($Y = 0, 22, 112$). To determine the effects on the endogenous metabolome in a dose-dependent manner, PLS-DA and OPLS-DA models were performed to compare the two drug treatment groups.

2.7.2. Dynamic model

PLS-based batch processing (PLS BP) modelling was performed only on the urine data set. A three-way matrix (X) was constructed, consisting of the time points (time variable, $Y = 3$), the observations ($N = 54$, for each batch or rat) and the NMR variables (spectral

integral regions, $K \sim 21k$). This method explains the greatest variance in the data set with respect to time. For the lower level model, control urine data was used to define batch normality and to generate control limits for the evolution of good batches corresponding to the average scores (± 2 and ± 3) of standard deviation (SD) from the mean trajectory of the urine samples, taking into account normal changes in the urinary metabolites due to diurnal, fasting, refeeding, hormonal and stress-related effects throughout the study. The data set corresponding to the venlafaxine treated rats were then mapped individually onto this control model to establish the metabolic deviations from the control group. The average scores, the control limits and the DModX (the residual distance to the model with 95% of critical significance level, $DCrit = 0.05$) were used to indicate deviations from the control model for each of the venlafaxine treated rats. The upper level model was developed to determine the correlation between the three-way matrix (X, as aforementioned) and the dose ($Z = 0, 22, 112$) using a PLS analysis to generate scores and loading plots, which explained the greatest variance in the data with respect to time and dose simultaneously [5,6,49]. To investigate the dose-dependent effect, the PLS BP modelling was performed (as aforementioned) only on the urine data set from the venlafaxine treatment groups, and batch normality was established by the low dose group.

Plots of the PLS BP scores for the two vectors ($t[1]$ and $t[2]$) were mapped to generate a time-course trajectory plot describing the different phases of the dominant metabolic perturbations with time. The xenometabolic trajectory plot was calculated using the mean PCA score values of the low dose and high dose urinary spectral data set.

2.8. Variable selection

The regression coefficients from the PLS, OPLS and upper level PLS BP models were divided by the jack-knife interval standard error to give an estimate of the t-statistic. Variables with a $|t\text{-statistic}| \geq 1.96$ (z-score, corresponding to the 97.5 percentile) were considered significant [51]. The corresponding loadings were back-transformed in Excel (Microsoft, USA) and plotted with the colour-coded value of the t-statistic of the variables in MatLab. For the lower level PLS BP, only the variables (spectral integral regions) for the batches which were found outside the model tolerance were established from the contributions plot and those with a $|SD| \geq 2$ were considered significant. Important changes were defined by the significant variables detected by both the stationary and dynamic models. Statistical changes were supported by visual examination of the spectra, and changes in the metabolites were calculated relative to the levels of the control group at each time point.

2.9. Antimicrobial activity test

Antimicrobial susceptibility testing was performed using the disc diffusion assay [52]. Plates were inoculated with a standardized suspension (0.5 McFarland scale) of the tested strain. *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC-25923 were used in this study. The sterile antimicrobial disks were soaked with a solution of the test drug and placed onto Mueller Hinton Agar (BD Difco, US). The plates were incubated at $35 \pm 2^\circ\text{C}$ for 16 to 18 h before measuring the size of the inhibition zone, which is correlated with the antimicrobial activity of the drug. ($\pm$)-Venlafaxine hydrochloride, ($\pm$)-venlafaxine and ($\pm$)-desvenlafaxine (as free bases) were tested and their antimicrobial activity was compared with that of ceftriaxone. This test was done in triplicate using two quantities of the drug (40 and 120 μg).

3. Results and discussions

There are many different types of antidepressants used to treat depression and other behavioural disorders, including ($\pm$)-venlafaxine and its major active metabolite, ($\pm$)-desvenlafaxine, which is prescribed under the monoamine theory. Their mechanism of action is related to the potentiation of serotonin (5-HT), norepinephrine (NE), and to a lesser extent dopamine (DA) by inhibiting the respective transporters, thus increasing the bioavailability of these neurotransmitters at the synapse. However, the exact mechanisms of action are still unknown, as is the case with other types of antidepressants and more generally with the etiology and pathology of depression. Moreover, the reason for the time lag of response to treatment is also unclear, although it is related to pre- and post-synaptic adaptive mechanisms and/or neurogenesis [1].

3.1. Animal model

($\pm$)-Venlafaxine hydrochloride was administered at therapeutic doses. Selection of the doses was based on preclinical studies in which the action of this antidepressant was demonstrated [38]. These doses were under the LD50 reported for female rats (350 mg/kg b.w.). The study was conducted with the untargeted NMR-based pharmacometabonomic approach in which different metabolites were identified in urine and faecal water. Only some of these metabolites showed changes derived from ($\pm$)-venlafaxine treatment (Supplementary Table S1 and Figures S1-S3). The advantage of this approach in the present context is that the rat body is considered a supraorganism, which allows for the detection of disrupted pathways, including the mammalian-microbial combinatorial metabolism that is finally reflected in the urine and faecal metabolotypes at the time points of the analysis. This aim is useful for generating hypothesis about the effects of ($\pm$)-

venlafaxine (and likely those of its major active metabolite (±)-desvenlafaxine) in the gut-microbiome-brain-liver-kidney-immune-cardiovascular system axis (GMBLKICA).

3.2. Xenobiotic metabolites

After oral administration, (±)-venlafaxine is metabolized via first-pass metabolism to yield (±)-desvenlafaxine and the rest of its metabolites, which are primarily excreted in urine [39]. Therefore, the signals of the venlafaxine xenometabolome were removed from endogenous metabolic data to allow for reliable biological interpretation. The chemical shifts and multiplicity of the venlafaxine-derived compounds were confirmed by using information from different sources: 1) 1D and 2D spectra of some standards (Supplementary Table S2), 2) 1D ^1H NMR spectra from treated groups at the first point in time, in which the predominant signals were from venlafaxine xenometabolome (Supplementary Figure S4A), whose intensities were proportional to the dose, and 3) 2D spectra from representative urine samples of the higher dose group at the first time points (Supplementary Figure S5). With this information, a singlet with $\delta\ ^1\text{H} = 2.828$ ppm was observed, which corresponds to the $\text{N}(\text{CH}_3)_2$ fragment of the venlafaxine-derived compounds. Since this did not overlap with drug or endogenous metabolite signals, it was used as a driver peak to run the STOCSY algorithm for the purpose of removing drug-related peaks (Supplementary Figure S4B).

Regarding the drug vehicle, the driver peak was a singlet with $\delta\ ^1\text{H} = 3.712$ ppm, which corresponds to the sorbitan moiety. This chemical shift was confirmed by a direct 1D ^1H NMR spectrum of the vehicle (5% Tween-80 w/v; Supplementary Figure S6), and by reports from the literature [4].

3.3. Chemometric data analysis

3.3.1. Stationary model

PCA analysis was performed on the datasets of urine and faecal water, observing that clustering by dose is more evident in urine than in faecal water (Supplementary Figures S7-S8). Comparisons among the three groups were performed using PLS and OPLS. To determine the dose-dependent effect, pairwise comparisons between the low-dose and high-dose groups were carried out with PLS-DA and OPLS-DA. The R^2 and Q^2 values as well as the results of the validation methods (permutation test and CV-ANOVA) for all the models are summarized in Supplementary Tables S3-S4. For urine, all the models were significant, while for faecal water only the pairwise comparison models between control and high-dose group were significant. Regarding faecal water, there was a clear separation between the control and high-dose groups in their cross-validated scores plots,

and this behaviour was due to the metabolic alterations shown in the corresponding loading plot (Figure 1). Even though the major change observed was in urine, the information obtained from faecal water was useful to confirm the effect of treatment in the gut microbiome.

3.3.2. Dynamic model

The lower level BP PLS model produced from the control group dataset had two components, describing 34% of the variation in the average data (X) and 92% of the time variation (Y). According to cross-validation, the model was able to predict 87% of the variation in Y, indicating a relationship with time. From this model was generated the batch process scores plot, which indicated consistency with control urine. However, the trajectory showed slight changes over time, which corresponded to normal physiological variation derived from diurnal, fasting, refeeding, and probably hormonal effects (Supplementary Figure S9A). These changes were observed using the dynamic model via lower level PLS BP, from which all batches (rats) were under the control limits, representing a “well-behaved” group, to be compared with the rats from treated groups. The BP scores plot and the DModX plot generated for the test set of the (±)-venlafaxine treated group were mapped onto the control model (Supplementary Figures S9B-S10A), and both the individual animal trajectories and the mean group trajectory showed deviations from the ‘normality’ described by the training set. The variables that generated these deviations are described in Table 1.

To study the dose-dependent effect, a new model was constructed (as aforementioned) with a new “well-behaved” group from the low-dose group dataset to establish control limits for comparing the behaviour of the batches (rats) of the high-dose group. This model comprised two components that described 33% of the variation in the average data (X) and 91% of the time variation (Y). The model was able to predict 85% of the variation in Y according to cross-validation. The test set was mapped as aforementioned, with the BP scores plots and the DModX plot shown in Supplementary Figures S10B-S11, and the variables which determined the deviation from the ‘normality’ summarized in Supplementary Table S5.

3.3.2.1. Normal changes

The diurnal and hormonal effects observed in urine levels of citrate, succinate, 2-oxoglutarate, taurine, *trans*-aconitate, dimethylglycine, glycine, creatine, creatinine, hippurate, glucose, TMAO and the NACs (N-acetyls of glycoproteins) are in accordance with the literature [5,6,53]. Moreover, the fasting and refeeding effects produced a urinary

metabotype characterised by both host and sym-xenobiotic metabolites derived from the ingredients contained in the rodent laboratory chow 5001. These metabolites are 1-methylnicotinamide, trigonelline, 3-(3-hydroxyphenyl)propionate (m-HPPA), 3-indoxylsulfate (3-IS), 4-hydroxyphenylacetate (4-HPA), hippurate, hydrocinnamate, formate, dimethylglycine, dimethylsulfone, TMAO, phenylacetyl glycine, *p*-cresyl glucuronide, creatine, methylhydantoin, proline betaine, isovaleryl glycine, leucine, pyruvate, pantothenate and pyridoxine (Supplementary Table S6) [54,55]. Since the urine from female rats is more varied in composition than that of males, as a result of the influence of hormonal changes [53], this normal physiological variation as well as diurnal, fasting and refeeding effects were removed from the real changes derived from the drug treatment using PLS BP modelling. Also, if the stress-related effect is present throughout the experiment, this model has the property of removing it [6]. In respect to the lower level BP PLS model constructed with the low-dose data, its 'normal behaviour' result was similar to that of the control group, with the exception of the absence of changes in urinary levels of 1-methylnicotinamide, 3-indoxylsulfate and U1, but with the presence of OAC1 (Supplementary Table S6). This behaviour may be related to the effect of ($\pm$)-venlafaxine in the urinary metabotype.

3.4. Effect of ($\pm$)-venlafaxine hydrochloride in urinary and faecal metabolotypes

The endogenous metabolic trajectory was indicative of homeostatic perturbation, which was most notable from 6-12 h after administration of a single dose of ($\pm$)-venlafaxine hydrochloride, followed by recovery of metabolic homeostasis by 24 h post-dosing (Figure 2A). The disruption at the first two points in time can be related to the respective dose remaining in the rat (FDR = $\sim$ 3.1%, $\sim$ 0.1%), and the recovery at 24 h to the complete excretion of the drug by the kidneys (FDR = 0.0001%). This same behaviour at the first two points in time is observed in the ($\pm$)-venlafaxine trajectory and reflects the presence of its metabolites, followed by their complete clearance by 24 h post-dosing that was detected in urine by 1D ^1H NMR (Figure 2B).

In order to improve the biological interpretation of the results and to reveal the effect of ($\pm$)-venlafaxine, statistical filtering was performed, which consisted of selecting the metabolites detected by both the stationary and the dynamic models (Tables 1-3). This procedure was also applied to the models used to explore the dose-dependent effect. To compare the differences between both models, cross-validated scores plots were obtained. The disruption of metabolite urinary levels in the loadings demonstrated a clear separation

between the three groups at the three time points. However, only the disruption corresponding to the first point in time is shown because at this time the greatest effect of (±)-venlafaxine was observed (Figure 3).

(±)-Venlafaxine hydrochloride treatment caused the depletion of creatinine, dimethylamine (DMA), formate, hippurate, methylamine, N-acetyl glycoprotein 3 (NAC3), proline betaine and phenylacetylglutamate (PAG). The decreased level of hippurate was found to be a dose-dependent effect (Table 3 and Supplementary Tables S5 and S7).

Although the drug treatment-induced metabolic changes in faecal water were minimal compared with those in urine, a decrease in some gut microbiota-related metabolites, including 3-(3-hydroxyphenyl)propionate (m-HPPA), acetate and propionate, was observed only in the faecal water and only in the high-dose group. The drug vehicle, on the other hand, was detected only in the faecal water samples, consistent with previous reports that tween 80 is innocuous to urinary and faecal metabolites [4,7].

Hippurate, PAG, m-HPPA, formate, acetate, propionate, DMA and MA are known to be produced by combinatorial metabolism between the host and the gut microbiome [16,24,31,54]. It is for this reason that a decreased level of these co-metabolites suggests a significant involvement of the gut microbiome in response to (±)-venlafaxine, likely caused by its most active metabolite, (±)-desvenlafaxine.

In this context, animals treated with antibiotics have been reported to show a reduction in the excretion of co-metabolites such as hippurate, PAG, TMAO and SCFAs [4,32]. Hence, the possible antimicrobial activity of (±)-venlafaxine and (±)-o-desmethylvenlafaxine against gram positive and gram negative bacteria was explored *in vitro*. Both drugs were found to be completely ineffective against the microorganisms tested. These observations are consistent with previous *in vitro* experiments using non-antibiotic drugs (including antidepressants) tested against a number of microorganism, in which (±)-venlafaxine hydrochloride was ineffective but selective serotonin reuptake inhibitors (SSRIs) exhibited appreciable antimicrobial activities [52]. Therefore, these results suggest that (±)-venlafaxine and (±)-desvenlafaxine do not alter the composition of the gut microbiota.

Nevertheless, these antidepressants may disrupt the bidirectional communication in the gut-microbiome-brain axis and therefore perturb the normal physiology of the virtual organ. This would then be reflected in the fluctuations of the co-metabolite levels in urine and faecal water post-treatment. In this regard, the gut microbiome is involved in the

modulation of mood and behaviour. For instance, decreased urinary levels of hippurate and PAG have been detected in children with autism [31]. In addition, the gut microbiome is disrupted in animal models of depression [17], and normal mice treated with antibiotics have shown changes in behaviour [18]. Nonetheless, in germ-free mice no changes have been observed [26].

Thus, (±)-venlafaxine and (±)-desvenlafaxine affect the brain, and based on our results they may affect the gut microbiome and therefore the gut-brain axis. However, due to the complex nature of this axis, it is difficult to elucidate the exact mechanisms of action of a drug that involve changes in the brain and the gut microbiome. Moreover, it is known that drugs have on-target and off-target activities [34,36] through reactome pathways that form the drug-target interaction network [35], and these activities are related to therapeutic and adverse effects [33,37]. (±)-Venlafaxine and (±)-desvenlafaxine may not be the exception, as their reactome pathways related to their on-target activity are comprised of serotonin, norepinephrine, and to a lesser extent dopamine transporters in the brain (SERT, NET and DAT respectively, the binding Database, <http://www.bindingdb.org/>). However, these transporters are also expressed in other cells outside of the CNS [23,30], meaning that these drugs can affect them via an off-target effect. Moreover, based on the perturbation of co-metabolites observed in the current study, the gut microbiome may be affected as well. Nonetheless, due to the complexity of the supraorganism (the rat body) response to (±)-venlafaxine treatment, the (±)-venlafaxine network of interactions between the gut microbiome and host, which are related to its therapeutic and adverse effects and may be disrupted by on-target and off-target effects, are integrated from different sources and summarized in Scheme 1.

The gut microbiome is a virtual organ with endocrine capacity that is embedded in the brain-gut-microbiome axis, and it is in a constant bidirectional communication to maintain homeostasis. (±)-Venlafaxine and (±)-desvenlafaxine may disrupt some microbiota signalling pathways. Firstly, gut microbiota can produce neurotransmitters such as serotonin (5-HT) and norepinephrine (NE), and can regulate 5-HT levels in the brain via production of tryptophan, which is able to pass the blood brain barrier (BBB). All these pathways have an effect not only in the CNS but also in the ENS [15-17,22]. Secondly, SCFAs modulate the immune response in the intestine via T cells, macrophages and dendritic cells [21]. The latter can send signals to the brain via the vagus nerve in response to gut microbiota signalling, and also are sources of energy in the gut [18,19]. Moreover, SCFAs are able to pass the BBB. In particular, acetate has effects in hypothalamic

neuronal activity related to appetite regulation [22]. Thirdly, the gut microbiome produces trace amines, such as tryptamine, that modulate mood [20,22]. Tryptamine inhibits the activity of 5-HT and dopamine (DA) in the brain and has been related to a number of mental disorders such as depression [22,56].

Regarding the other side of bidirectional communication, the capacity of (±)-venlafaxine to increase the bioavailability of 5-HT and NE (the on-target effect) may exacerbate their neuroendocrine activity not only in the brain but also in peripheral cells that express receptors to these neurotransmitters. In this regard, the modulation of mood and behavior has been related to the serotonergic pathway, which is under the influence of the gut microbiome [17]. Enterochromaffin cells (ECs), the main source of 5-HT, can secrete this neurotransmitter on both the basolateral and luminal side. Thus, these cells act as bidirectional signal transducers between the host and gut microbiome [24,27]. This neurotransmitter is also found in enteric neurons and is involved in the regulation of intestinal motility. Co-metabolites such as SCFAs are able to modulate production of 5-HT in ECs, and the gut microbiome is thereby able to stimulate the enteric nervous system and regulate intestinal motility [15,22,27,57].

After carrying out its activity, the serotonin transporter (SERT) terminates serotonergic signalling. This transporter is not only located in the brain but also in enterocytes, enteric neurons and platelets [23,30]. Serotonin produced by ECs has many functions aimed at maintaining homeostasis, such as secretion of other bioactive molecules, peristalsis, vasodilatation, nociception and nausea via the vagus nerve [17,19]. Additionally, gut-derived serotonin inhibits bone formation [58].

On the other hand, (±)-venlafaxine/(±)-desvenlafaxine may possibly exert an off-target effect in the cardiovascular system. By inhibiting SERT in platelets, SSRIs reportedly decreased platelet aggregation, a protective effect against cardiovascular disease [10]. Another point of view is that this effect on platelets can be related to adverse effects in the cardiovascular system (Pfizer, www.pfizer.com).

In addition, immune cells such as lymphocytes, monocytes, macrophages and dendritic cells have 5-HT receptors that play a role in gut inflammation, an effect that has also been related to changes in mood and behaviour. Moreover, mastocytes, macrophages and T cells can produce 5-HT from tryptophan, and this amine acts as a chemotactic factor for eosinophils, dendritic cells and mastocytes [30]. Hence, 5-HT is important in the modulation of immune responses. Accordingly, in gut diseases such as IBS and IBD

serotonergic signalling is disrupted not only in the CNS but also in the ENS [23,24,30]. Furthermore, SCFAs have exerted potent anti-inflammatory functions in mouse IBD models [25], and in IBD patients the comorbidity of depression and anxiety has been observed [18]. In another animal model of inflammation, 5-HT levels and the number of ECs were found to be increased, while SERT was downregulated. Therefore, the amplification of serotonin activity in the gut produces inflammation [17].

In addition, mice treated with paroxetine (an SSRI) to inhibit SERT in the GI tract, produced a reduction in peristaltic activity that resulted in constipation [59]. Therefore, (±)-venlafaxine and (±)-desvenlafaxine (SNRIs) can exert their effects not only in the CNS but also in the ENS. Thus, the decrease in levels of SCFAs observed presently in faecal water metabotype suggests a relationship between the aforementioned mechanisms and the adverse effects of (±)-venlafaxine/(±)-desvenlafaxine involving the digestive system, including anorexia, constipation, diarrhoea, nausea and vomiting (Pfizer, www.pfizer.com).

Perturbation in 5-HT levels has also been related to a systemic increase in proinflammatory cytokines such as IL-1 β and IL-6. These cytokines activate the HPA axis, which is involved in the control of stress and in the regulation of mood and emotions [60]. The HPA axis also has a direct effect on the intestines via bidirectional communication with the ENS, CNS, immune system and gut microbiome [15,17]. In addition, patients with depression have shown increased serum levels of IL-1 β and TNF- α , which in turn produce 5-HT reuptake that upregulates SERT. Therefore, levels of this neurotransmitter are decreased at the synapse [61], and at the same time these proinflammatory cytokines diminish the expression of the 5-HT_{1A} receptor in postsynaptic neurons [22]. Hence, there is a relationship between the brain and the immune system that is involved in the alteration of mood and behaviour. Since O-acetyl and N-acetyl glycoproteins are involved in inflammatory conditions [62], the effects observed in the urinary levels of OAC1 and NAC3 can be related to the perturbation in the GI tract derived from the increased levels of 5-HT resulting from inhibited uptake.

Norepinephrine (NE) can exert an anti-inflammatory activity in the GI tract by inhibiting the activity of NK cells and macrophages [63]. The functions of the GI tract are regulated by its own nervous system (the ENS), enteric glia and enteroendocrine cells (including ECs), the gut connectome (which is in close proximity and continuous communication with the CNS via the vagus nerve) [14,64], the immune system, and the gut microbiome [26]. Working together, these systems and factors exert important functions to maintain homeostasis in

the GI tract, such as the regulation of intestinal motility, the modulation of nutrient absorption, and the maintenance of the integrity of the intestinal mucosa barrier [65]. In this gut connectome there are serotonergic, norepinephrergic and dopaminergic neurons [23] that are also involved in the modulation of mood and behaviour. In this manner, the cycle of entrance and reuptake of 5-HT, NE and DA has to be intact to maintain normal physiology in the GI tract [59] and brain. However, this cycle can be disrupted by (±)-venlafaxine/(±)-desvenlafaxine, thus exerting an effect in all bidirectional communication in the multiorgan axis. In addition, quorum-sensing molecules, used by microbes to communicate with each other, are also recognized by host cells and may influence enteroendocrine cells, immune cells, and neurons in the gut [28]. NE released into the gut lumen can activate adrenergic-like QseC (quorum-sensing sensor kinase) receptors on the surface of the gut bacteria with particular relevance for gut-brain interactions during stress [27].

The greatest amount of creatinine is in skeletal muscle and is an energy source in this tissue. Creatinine mainly derives from diet or *de novo* synthesis by the kidney-liver axis [66]. Additionally, some intestinal bacteria can produce it or degrade it into methylamine [54]. Creatinine and proline betaine are organic osmolytes that are related to osmoregulation by binding to osmoreceptors in the CNS or GI tract, and also can exert effects in renal physiology [55,66]. The decrease of urinary levels of these osmolytes and methylamine can be ascribed to an effect of (±)-venlafaxine/(±)-desvenlafaxine in osmoregulation via the gut-brain-kidney axis.

The time lag in the therapeutic effect of (±)-venlafaxine (which can be extended to SSRIs) may be related to several signalling pathways that can be disrupted by this drug in the multiorgan axis. Hence, there may be a readaptation process not only in CNS but also in all the elements that comprise the axis. In this context, it has been reported that intestinal bacteria modulate the plasticity of serotonergic and GABAergic neurons in the CNS [26].

4. Conclusions

It has been herein demonstrated that a single dose of (±)-venlafaxine hydrochloride induced alterations in urinary and faecal water metabolites in normal rats. This may result from the on-target and off-target effects of (±)-venlafaxine hydrochloride by altering the continuous bidirectional communication inside the gut-microbiome-brain-liver-kidney-immune-cardiovascular system axis (GMBLKICA). Such alterations may be involved in the therapeutic and adverse effects of this drug, as well as the lag period for response to

treatment. The complexity of the supraorganism, together with its diseases and their treatments, can make it difficult to elucidate the exact mechanisms of action of a drug and the etiology and pathology of diseases. Therefore, the integration of a classical pharmacological approach with multi-omics analysis in preclinical and clinical trials can be of great benefit. Regarding animal models, it is convenient to use normal animals to reveal pathways disrupted by a drug, and thus detect adverse effects and/or secondary therapeutic activities.

5. References

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6. Figure captions

Figure 1. The OPLS-DA cross-validated scores plot (left) and loading plot (right), derived from faecal water 1D ^1H NMR spectra, show the difference between the control group (green) and venlafaxine-treated group (red), which was given 112 mg/kg b.w. Key: 6, 3-(3-hydroxyphenyl)propionate; 9, acetate; 52, propionate; 66, U4.

Figure 2. The mean PLS BP trajectory plots for endogenous metabolic data set (A) and venlafaxine xenometabolome data set (B). Error bars represent the standard error for the average value of each time point. Key: control group (green), venlafaxine-treated groups given 22 mg/kg (blue) or 112 mg/kg (red).

Figure 3. (A) The OPLS cross-validated scores plot (left) and loading plot (right), and (B) upper level PLS BP cross-validated scores plot (left) and loading plot (right) derived from urinary 1D ^1H NMR spectra for the time point at 6 h. These graphs show the difference between the control group (green) and venlafaxine-treated groups, which were given 22 (blue) and 112 (red) mg/kg b.w. Coloured by t-statistic. Key: 21, creatinine; 22, dimethylamine; 30, hippurate; 40, methylamine; 44, NAC3; 47, p-cresyl glucuronide; 49, phenylacetyl glycine; 51, proline betaine; 53, pyridoxine.

Scheme 1. ($\pm$)-Venlafaxine hydrochloride (($\pm$)-V HCl) network with partial visualization of the direct and indirect interactions of the gut-microbiome-brain-liver-kidney-immune-cardiovascular system axis (GMBLKICA) that have multiple targets. After oral administration, this drug reaches the gut lumen, most likely as a ($\pm$)-venlafaxine free base (($\pm$)-V). Then it undergoes first-pass metabolism that yields ($\pm$)-desvenlafaxine (($\pm$)-OV, its most active metabolite) and the rest of the metabolites that comprise the xenometabolome (detected only in urine). ($\pm$)-V HCl produces changes in both urinary and faecal metabolotypes, decreasing levels of osmolytes and gut microbiota co-metabolites (GMC, see text for details), which suggests an effect on the kidney and the gut microbiome. ($\pm$)-V and ($\pm$)-OV inhibit the transporters of serotonin (SERT), norepinephrine (NET), and to a lesser extent dopamine (DAT) in the central nervous system (CNS), and very likely in enterocytes, platelets and enteric nervous system (ENS), via on-target and off-target effects. Outside the CNS, enteric neurons and gut microbiota produce serotonin (5-HT),

norepinephrine (NE) and dopamine (DA). Moreover, the main source of 5-HT is enterochromaffin cells (ECs) in the gut. Since (±)-V and (±)-OV inhibit the uptake of these neurotransmitters, their bioavailability is increased. Therefore, by their potentiation activity (not only in the brain but also in the enteric nervous system, gut microbiome, immune system, platelets and bone), they may exert different biological outcomes related to modulation of mood and behaviour (therapeutic) as well as adverse effects. Key: (±)-V HCl, (±)-Venlafaxine hydrochloride; (±)-V, (±)-venlafaxine free base; (±)-OV, (±)-desvenlafaxine; (±)-OVG, (±)-desvenlafaxine glucuronide; UGT, UDP-glucuronosyl transferase; CYP450, either CYP2D6 or CYP3A4; VX, venlafaxine xenometabolome; GMC, gut microbiota co-metabolites; 5-HT, serotonin; NE, norepinephrine; DA, dopamine; SERT, serotonin transporter; NET, norepinephrine transporter; DAT, dopamine transporter; ENS, enteric nervous system.

Figure 1

Figure 2

Figure 3

Scheme 1

Graphical abstract

Table 1. Effects of (±)-venlafaxine in urinary metabotype according to the dynamic model.

Metabolite	Time point (h). Lower-level PLS BP					Upper-level PLS BP ^c n = 36		
	6 n = 32	12 n = 28	24 n = 14	Global ^a n = 74	Complete batches ^b n = 11	6	12	24
3-(3-hydroxyphenyl)propionate	-	-	-	↓	-	-	-	↓
3-Indoxylsulfate	-	-	-	-	-	↓	-	-
4-Hydroxyphenylacetate	-	-	-	-	↓	-	-	-
Acetate	-	↓	-	-	-	-	↓	-
Betaine	-	-	-	-	↓	-	-	-
Carnitine	-	-	-	-	-	-	↓	-
Creatine	-	-	-	↑	-	-	-	↑
Creatinine	↓	-	-	↓	↓	↓	-	-
Dimethylamine	↓	-	-	-	-	↓	-	-
Dimethylglycine	-	-	-	-	↓	-	-	-
Formate	↑	↓	-	↓	↓	-	↓	-
Hippurate	↓	-	-	↓	↓	↓	-	↓
Hydrocinnamate	-	-	-	-	-	↑	-	↑
Isovalerylglycine	↓	-	-	-	-	-	↓	-
Leucine	-	-	-	-	-	-	↓	-
Methylamine	↓	↓	↓	↓	↓	↓	↓	-
Methylhydantoin	-	-	-	-	↓	-	-	-
NAC2	↓	-	-	-	-	-	↓	-
NAC3	↓	-	-	↓	↓	↓	-	-
OAC1/2	-	-	-	-	-	-	↓	-
<i>p</i> -Cresyl glucuronide	↓	-	-	↓	↓	↓	-	-
Phenylacetyl glycine	↓	-	-	↓	↓	↓	-	-
Proline betaine (Stachydrine)	↓	↓	↓	↓	↓	↓	↓	-
Pyridoxine	↓	-	-	-	↓	↓	-	-
Pyruvate	↓	-	-	↓	↓	↓	-	-
Succinate	-	-	-	-	↓	-	-	-
Taurine	-	-	↓	-	-	-	-	-
<i>Trans</i> -aconitate	-	-	↓	-	-	↓	-	-
TMAO	-	-	-	-	↓	-	↓	↑
U2	-	-	-	-	-	↓	-	-
U3	-	-	-	-	-	-	-	↑

^aGlobal refers to all deviated urinary samples from the control limits at any given time point.

^bComplete batches correspond to the rats (batches) out the control limits at the three time points.

^cBoth venlafaxine-treated groups were included. ↑, above control levels; ↓, below control levels.

Table 2. Effects of (±)-venlafaxine in urinary metabotype according to the stationary model.

Metabolite	Time point (h)					
	6		12		24	
	PLS	OPLS	PLS	OPLS	PLS	OPLS
3-(3-hydroxyphenyl)propionate (m-HPPA)	-	-	-	-	↓	↓
3-Indoxylsulfate (3-IS)	↓	-	-	-	-	-
4-hydroxyphenylacetate (4-HPA)	-	-	↓	-	↓	↓
Acetate	-	-	↓	↓	-	-
Carnitine	↓	-	-	↓	-	-
Creatinine	↓	↓	-	-	-	-
Dimethylamine (DMA)	↓	↓	-	-	-	-
Formate	-	-	↓	↓	-	-
Hippurate	↓	↓	↓	-	↓	↓
Hydrocinnamate	-	-	-	-	↑	↑
Isovalerylglycine	↓	-	-	-	-	-
Leucine	↓	-	-	-	-	-
Methylamine	↓	-	↓	↓	-	-
NAC2	↓	-	-	-	-	-
NAC3	↓	↓	↓	-	-	-
OAC1	-	-	-	↓	-	-
<i>p</i> -Cresyl glucuronide	↓	-	↓	-	-	-
Phenylacetylglutamate (PAG)	↓	↓	↓	-	-	-
Proline betaine (stachydrine)	↓	↓	↓	-	↓	↓
Pyridoxine	↓	↓	-	-	-	-
Pyruvate	↓	-	-	-	-	-
Succinate	-	-	-	-	↑	-
<i>Trans</i> -aconitate	-	-	↓	-	-	-
Trimethylamine N-oxide (TMAO)	-	-	↓	-	-	-
U1	-	-	-	-	-	↓
↑, above control levels; ↓, below control levels.						

Table 3. Summary of the urinary metabolites perturbed by (±)-venlafaxine, and the dose-dependent effects^a shown after statistical filtering.

Metabolite	Control vs venlafaxine ^b		Venlafaxine low dose vs high dose ^c	
	Time point (h)			
	6	12	6	12
Creatinine	↓	-	-	-
Dimethylamine (DMA)	↓	-	-	-
Formate	-	↓	-	-
Hippurate	↓	-	↓	-
Isovalerylglycine	-	-	-	-
Methylamine	-	↓	-	-
NAC3	↓	-	-	-
Phenylacetylglutamine (PAG)	↓	-	-	-
Proline betaine (stachydrine)	↓	-	-	-

^aChanges detected in the stationary model (PLS and OPLS) and the dynamic model (lower and upper level PLS BP). ↓, below control levels^b or low dose levels^c.

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