Drug-Induced Impairment of Mitochondrial Fatty Acid Beta-Oxidation: A Metabonomic Evaluation in Rats

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Abstract

Drug-Induced Liver Diseases (DILI) is frequent and potentially harmful. Steatosis, an accumulation of triglycerides in hepatocytes, is a DILI that may be fatal in the long term. It progresses insidiously and, due to the lack of obvious symptoms, is barely anticipated. During lead optimization in drug development, it is crucial to screen steatogenic molecules as early as possible. Unfortunately, to date, the detection of steatosis in animals can only be histopathologically ascertained. The present study aims to establish a non-invasive urine metabolic signature of nascent steatosis in rats exposed to some selected steatogenic model molecules. Metabonomics perfectly meets this expectation and also provides a dynamic assessment of the disease. In vivo, rat urine fractions can indeed be collected over time in metabolic cages and analyzed by proton NMR spectroscopy. This approach, combined with chemometric tools, can give important mechanistic information on the biochemical pathways which are possibly altered by the tested molecules. In the present study, three prototypical compounds (valproic acid, tetracycline and dimethylformamide) were evaluated by metabonomics in Wistar Han rats to monitor the biochemical changes caused by the early development of microvesicular steatosis in the first 24 hours following exposure. A metabonomic pattern reflecting the consequences of the inhibition of the beta-oxidation pathway emerged, mainly evidencing trouble with energy production as well as in nitrogen metabolism.

Introduction

Steatosis, characterized by the accumulation of Triglycerides (TG) in hepatocyte vacuoles, is one of the most common Drug-Induced Liver Injuries (DILI) [1]. Various cellular mechanisms contribute to the development of steatosis, such as increased lipogenesis, decreased catabolism of Fatty Acids (FA) due to a default in mitochondrial and/or peroxisomal oxidations, or impaired synthesis and secretion of lipids and lipoproteins [2]. Among these mechanisms, the most frequent cause of xenobiotic-induced fatty liver is the alteration of mitochondrial functions including the inhibition of beta-oxidation (β-ox).

Mitochondrial β-ox is the major metabolic pathway for the degradation of fatty acids, which would otherwise eventually accumulate in hepatocytes in the form of lipid droplets. These vacuoles can take two different aspects: in microvesicular steatosis, the droplets are numerous but too small to move the nucleus in the cell, while in macrovesicular steatosis, the vacuoles are so large that they displace the nucleus [2].

When steatosis is due to an inhibition of β-ox, without involving any reshaping of proteins, it rapidly develops after exposure to the implicated drug. Although it can go unnoticed, and is virtually harmless at first, steatosis can become fatal with chronic exposure [3,4]. Therefore, in the early stages of drug development, it is essential to screen and discard molecules with a propensity to cause steatosis.

Some authors have evaluated the potential toxicity of various steatogenic xenobiotics [4,5]. The search for specific biomarkers of steatosis is on track but current methods of investigation are usually invasive and require a significant number of animals to dynamically evaluate the progression of the pathology.

Methods from the “omic” sciences offer new tools to explore cellular disorders [6-8]. As an example, 'H-NMR-based metabolomics allows the dynamic assessment of an evolving pathology from the spectroscopic analysis of biofluids collected over time. As such, it allows a reduction in the number of animals used during toxicology studies because interim sacrifices are no longer required [9]. This method has proved successful in assessing different types of toxicities from a bench of biofluids [9,10], biopsies [11], or cell extracts [12] collected from animals exposed to different prototypical toxic substances.

The present study then focuses on three steatogenic model compounds: Valproic Acid (VPA), Tetracycline (TC) and Dimethylformamide (DMF).

VPA is a branched fatty acid with a molecular configuration which is very similar to medium-chain fatty acids. Due to its GABA-like (β-Aminobutyric acid) effects and its inhibition of voltage-gated sodium channels, VPA is used as a broad spectrum antiepileptic drug [13]. More recently, promising effects in the treatment of some cancers have been attributed to VPA. It is indeed a potent inhibitor of Histone Deacetylase (HDACs) acting on the histones to promote the condensation of chromatin [14]. Unfortunately, VPA is bio activated into acyl-Co A that weakens the intramitochondrial pool of free Co A and reduces the oxidation of fatty acids. Moreover, a 4-valproate, one of its metabolites formed by the P450 cytochrome, undergoes β-ox to produce D2, Δ4-valproyl-Co A, an electrophile diene metabolite that inhibits different enzymes of the β-ox pathway [15].

Tetracycline is an antibiotic drug derived from Streptomyces and widely used against a large spectrum of bacteria. Fatal cases of microvesicular steatosis have been reported after repeated intravenous injections of high doses of tetracycline, or its derivatives [16]. The same was also noticed in laboratory animals. Its steatogenic effect results...
from two specific actions: an initial inhibition of β-ox [17,18] combined with a decreased hepatic secretion of VLDL (Very Low-Density Lipoproteins) [19].

DMF or N, N-dimethylformamide is an organic molecule derived from formic acid and di methylamine. It is primarily used as a solvent in the production of acrylic fibers, synthetic leathers and paintings, or as a catalyst in drug synthesis [20]. Its toxicity is dose-dependent and multiple. The people most affected by DMF are industrial workers who are regularly exposed to this solvent without wearing proper individual protection [21]. In addition, in cases of acute poisoning at high doses, DMF may also cause microvesicular steatosis, which can further lead to centrilobular necrosis after chronic exposure [22].

Knowing that it is essential to detect the onset of steatosis as soon as possible after exposure to drugs, the potential of metabonomics in screening steatogenic molecules will be evaluated. This primary goal is to identify a metabonomic signature characteristic of the early events of the disease, from which specific markers could be isolated. Such metabolic markers could then be further developed into biomarkers of drug-induced steatosis, but could also reveal new insights into the cellular mechanisms responsible for this adverse effect.

Results

The visual inspection of the 1H NMR spectra (Figure 1) already revealed major differences in the urine composition of pre-test and drug-exposed animals. To further investigate more subtle differences in the whole dataset acquired over time, multivariate data analysis was applied to the spectral data.

Figures 2-4 compares the 400 MHz 1H-NMR spectra of urine samples collected from pre-test rats and rats exposed either to VPA, TC or DMF respectively. The scores plots arising from the PLS-DA of reduced spectral data obtained from the urine of animals exposed to VPA (Figure 2A), TC (Figure 3A) or DMF (Figure 4A), illustrate some clear differences in the urine composition of pre-test and exposed groups (Figures 2B, 3B and 4B, respectively). In addition, the scores plot representing urine samples from VPA-exposed animals (Figure 2A) shows a clear separation between pre-test and 4h-8h, 12h-16h and 20h-24h after injection. In the cases of DMF and TC, even though some differences are still visible between unexposed and exposed animals, the time separation among samples is less obvious.

The corresponding loadings plots (Figures 2B, 3B and 4B) indicate spectral regions involved in the separation between pre-test and VPA-, TC-, or DMF-exposed animals respectively.
Specific changes observed in VPA-exposed rats

After exclusion of resonances arising from VPA and its metabolites, other discriminating signals, corresponding to intermediates of the endogenous metabolism (summary in Table 1), were identified. The PLS-DA (Figure 2A) picked up differences in the metabolic profiles of pre-test versus post-dose urine samples.

This model (Figure 2) also showed clear separations over time, as indicated by the comparisons between the pre-test and samples collected at 4h-8h, 12h-16h and 20h-24h post-injection. The samples collected at early time points (4-8h post-dose) are the most separated from the pre-test, while the composition of samples already started to recover towards pre-test values after 20-24h post-dose.

This separation was mainly due to reductions in many urine metabolites from VPA-exposed rats (Figure 2B). The list of VIPs (Table 1) indicated by the comparisons between the pre-test and samples collected of pre-test versus post-dose urine samples.

The PLS-DA highlighted some differences in the metabolic profiles of TC-exposed rats (Figure 3A), mainly due to a drastic drop in hippurate and several Krebs cycle intermediates (citrate, α-ketogluutarate and succinate) levels, as revealed by the corresponding loadings plot (Figure 3B). However, other cellular metabolites were excreted in the urine in greater amounts compared to controls, among which allantoin, taurine, creatinine, betaine and glycerol-3-phosphate presented the most significant changes. Also, urine fractions collected from 4 to 8h post-dose were characterized by increased allantoin and decreased citrate contents.

Specific changes observed in TC-exposed rats

DMF-exposed animals exhibited alterations in the urine levels of several metabolites including decreases in some TCA cycle intermediates; creatine, taurine and hippurate, when compared with pre-test samples (Table 1 for summary). Overall, all the discriminating metabolites revealed by the PLS-DA had a lower concentration in the post-dose urine fractions compared to the matching pre-test samples. Contrary to the analysis of the urine samples collected from VPA-exposed animals, time differences were more discreet in the case of DMF. The separation between treated animals and controls was still clear but the separation in the direction of the first principal component was mainly due to descriptors related to resonances arising from the drug and/or its metabolites. These descriptors were thus removed from the subsequent analyses. However, it has to be stressed that significant overlapping with resonances arising from endogenous compounds of biological interest was reported, jeopardizing the interpretation of the data.

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Discussion

The metabonomic study of the three model substances evaluated in this study revealed clear changes in the urine composition of rats after drug exposure. In addition, time differences in the urine metabolic trajectories of drug-exposed animals were also highlighted by the 1H-NMR-based metabonomic assessment, suggesting different modes of action to cause steatosis.

Specific changes observed in VPA-exposed rats

VPA mimics the fatty acids naturally present in the body and competes with coenzyme A (Co A), leading to an inhibition of β-oxidation [23]. It was observed that very few endogenous metabolites were more excreted in the urine of animals after exposure to VPA, compared to their matching pre-test urine, or to urine samples collected from control animals. Still, a slight increase in the concentration of succinate, clearly visible on a peak-to-peak examination but not in the loadings plot was noticed. This increase in succinate contrasts with changes observed for other Krebs cycle intermediates at 24h post-dose. Although

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**Table 1: Metabolic changes in exposed rats compared to pre-test rat.**

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shift</th>
<th>VPA</th>
<th>TC</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>1.92 ppm, s</td>
<td>▼ (0.62)**</td>
<td>▼ (1.9)*</td>
<td>-</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>2.29 ppm, s; 3.45 ppm, s</td>
<td>▲ (1.7)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adipate</td>
<td>1.56 ppm, m; 2.22 ppm, m</td>
<td>-</td>
<td>▲ (0.9)</td>
<td>-</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>2.45 ppm, t; 3.01 ppm, t</td>
<td>▼ (3.5)**</td>
<td>▼ (2.9)*</td>
<td>▼ (2.2)*</td>
</tr>
<tr>
<td>Allantoin</td>
<td>5.39 ppm, s</td>
<td>▼ (1.4)**</td>
<td>▲ (3.4)*</td>
<td>▼ (2.8)*</td>
</tr>
<tr>
<td>Betaine</td>
<td>3.27 ppm, s; 3.90 ppm, s</td>
<td>▼ (2.8)**</td>
<td>△ (0.7)</td>
<td>▼ (2.7)</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.54 ppm, d; 2.67 ppm, d</td>
<td>▼ (0.5)</td>
<td>▼ (3.1)</td>
<td>▼ (2.7)*</td>
</tr>
<tr>
<td>Creatine</td>
<td>3.04 ppm, s; 3.93 ppm, s</td>
<td>▼ (2.0)**</td>
<td>▲ (1.8)*</td>
<td>▼ (2.3)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.05 ppm, s; 4.06 ppm, s</td>
<td>▼ (1.5)**</td>
<td>▲ (1.9)*</td>
<td>▼ (1.6)*</td>
</tr>
<tr>
<td>Dimethylglycine (DMG)</td>
<td>2.93 ppm, s</td>
<td>▼ (0.8)**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fumarate</td>
<td>6.53 ppm, s</td>
<td>-</td>
<td>-</td>
<td>▼*</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.24 ppm, d</td>
<td>▼ (2.7)**</td>
<td>-</td>
<td>▼ (3.8)*</td>
</tr>
<tr>
<td>Glycerol-3-phosphate</td>
<td>3.61 ppm,m; 3.67 ppm, m; 3.82 ppm, m</td>
<td>-</td>
<td>▲ (1.9)*</td>
<td>-</td>
</tr>
<tr>
<td>Guanidinocacete</td>
<td>3.81 ppm, s</td>
<td>▼ (1.9)**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>3.97 ppm, d; 7.55 ppm, t; 7.64 ppm, t; 7.84 ppm, d</td>
<td>▼ (3.7)**</td>
<td>▼ (4.5)*</td>
<td>▼ (4.0)</td>
</tr>
<tr>
<td>Malate</td>
<td>2.39 ppm, dd; 2.69 ppm, m; 4.31 ppm, dd</td>
<td>▼ (1.1)**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-acetylglutamate</td>
<td>2.04 ppm, s</td>
<td>▼ (0.9)**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.40 ppm, s</td>
<td>△ (peak to peak)</td>
<td>▼ (1.4)*</td>
<td>▼ (1.8)*</td>
</tr>
<tr>
<td>Taurine</td>
<td>3.25 ppm, t; 3.43 ppm, t</td>
<td>▼ (2.6)**</td>
<td>▲ (3.8)*</td>
<td>▼ (3.1)*</td>
</tr>
<tr>
<td>Trans aconitate</td>
<td>6.6 ppm, s</td>
<td>-</td>
<td>▼ (0.9)*</td>
<td>▽ (0.40)</td>
</tr>
<tr>
<td>Trimethylamine Oxide (TMAO)</td>
<td>3.27 ppm, s</td>
<td>▼ (4.0)**</td>
<td>-</td>
<td>▼ (3.0)*</td>
</tr>
</tbody>
</table>

The direction of changes (indicated by the arrows) was determined from the respective position of the pre-test and post-dose samples in the scores and loadings plots. VIP values are shown in brackets. s=singlet; d=doublet; dd=double of doublets; t=triplet; m=multiplets. Wilcoxon matched-pairs signed-ranks test: *p-value < 0.05; ** p-value < 0.01
slight, the succinate increase suggests an alteration in the lipid metabolic pathways. This metabolite, in addition to being part of the Krebs cycle, is indeed the result of the ω-oxidation, an alternative biochemical pathway to β-ox [24]. This shift towards the second type of oxidation processes occurs in case of troubled trafficking across the mitochondrial membranes. The decrease in urine taurine may be linked to this switch to the lipid metabolism through its recruitment in the synthesis of taurocholic acid, a bile acid used by liver cells to facilitate the secretion of fat and some toxic compounds into the bile [25].

A significant amount of other metabolites was less excreted in urines from VPA-exposed rats, in particular, some Krebs cycle intermediates, like citrate and α-ketoglutarate, as well as malate, which are located either in the mitochondria or in the cytosol.

The interaction of VPA with the urea cycle has been repeatedly demonstrated [26,27]. In many studies, this effect was evidenced by the reduction in ornithine, suggesting an alteration of the urea cycle, but also by hyperammonemia, occurring in animals exposed to VPA [27,28]. Among others, Coulter et al., highlighted that hyperammonemias caused by VPA, either through the reduction of the hepatic N-Acetyl Glutamate (NAG) (reduced in VPA-exposed animals) or by affecting the available pool of acetyl-Co A [26,29]. The latter contributes, with glutamate, to the production of NAG, which is itself seen as an activator of carbamoyl phosphate synthetase I. The inhibition of this enzyme causes excessive release of ammonia that alters, among others, cell respiration by exhausting α-ketoglutarate. Moreover, this also induces a shift in the balance between glutamine and glutamate in the cells of the parietal lobes of patients treated with VPA [30].

The lower urine excretion of hippurate may indicate an alteration of the liver function since it is synthesized in liver cells by the conjugation of benzoic acid with glycin (also related to the metabolism of creatine) [31]. The decrease in guanidoacetate is also linked to creatine. Indeed, guanidoacetate a precursor to creatine, which is involved in the production of creatinine (also reduced in this study).

VPA is also known to consume glutathione [32], an effect that seems to be confirmed in this study by the decreased levels of Trimethylamine Oxide (TMAO), creatine, dimethylglycine and betaine, all precursors to this antioxidant molecule.

The onset of the fatty liver condition is also reflected in the urine composition by the decreased level of acetate, which is used to maintain the synthesis of cholesterol. The hmgcs1 enzyme involved in this pathway is upregulated and, in parallel, involved in the increase in acetoacetate [33], itself released by the Co A lyase.

Unlike the study of Schnackenberg L K., [34] which reported an increased glucosuria in pregnant mice exposed to VPA, glucosuria seemed to dramatically drop in the experimental conditions. This difference may be due to interspecies difference or to the fact that the rats were found to be quite lethargic during the first 4 hours following the injection of VPA and, therefore, considerably reduced their food consumption during this period.

**Specific changes observed in TC-exposed rats**

The steatogenic effect of tetracycline is known to result from the decrease in the mitochondrial β-ox of fatty acids and the reduction in the secretion of hepatic triglycerides, due to the slowdown activity of MTP (Microsomal Triglyceride Transfer Protein) [28]. It also promotes the activity of a series of enzymes involved in the synthesis of cholesterol and/or hepatocyte triglycerides causing additional lipid accumulation. Indeed, according to Yin H Q et al., the expression of GPD2 and AGPAT2 (genes involved in the formation of triglycerides) is increased in TC-exposed mice, which results in an excess of liver triglycerides. This effect was suggested in the study by the higher urine excretion of a metabolite that was assigned to glycerol-3-phosphate based on the corresponding resonances. This observation may corroborate the TC-induced higher expression of Glycerol-3-Phosphate Dehydrogenase (GPD2) [35].

TC also induced a reduction in the expression of Crat1 (Carnitine acyl transferase). This enzyme is involved in the formation of acyl-carnitine that is required to promote the transport of acyl-Co A through mitochondrial membranes. Consequently, its inhibition leads to reduced β-ox activity. This is rapidly balanced by microsomal ω-oxidation, an alternative pathway catalyzed by CYP4E. It has been shown that TC is able to increase the expression of two members of this cytochrome family, namely Cyp4f14 and Cyp4f18 [35]. Unfortunately, the resulting excess of diacetyloxy fatty acids (adipate increase in urine) can be toxic to the mitochondria, an adverse effect that contributes to liver and kidney damage, demonstrated in this study by the increased excretion rates of urinary taurine, creatine and creatinine. Such adverse effects have already been reported by others in mice and humans exposed to high doses of TC [17].

Finally, TC also acts on the synthesis of cholesterol by increasing the expression of Hmgcs1 (needed for the production of HMG-Co A) and depletes the pool of Co-A which can no longer be used for β-ox purposes [35]. These changes eventually harm Krebs cycle activity due to an inadequate supply in acetyl-Co A. This explains the drop in Krebs cycle intermediates observed in the urine of TC-exposed animals.

**Specific changes in DMF-exposed rats**

DMF is a solvent currently used in organic chemistry. It causes the depletion and alteration of mitochondrial DNA, increases the levels of serum SDH [29] and decreases the concentration of glutathione. It is also known to induce microvesicular steatosis following acute exposure [22].

DMF is primarily metabolized in the liver and its metabolites are rapidly eliminated through the kidney. The major metabolic pathway, which occurs via the P450-2E1 cytochrome, causes the hydroxylation of the methyl groups and leads to the urine excretion of N-(Hydroxymethyl)-N-Methylformamidamide (HMMF), HMMF can in turn generate either N-Methylformamidamide (NMF), further oxidized in N-(hydroxymethyl)-Form amide (HMF) which subsequently decomposes into N-methyl form amide, or N-acetyl-S-(N-Methylcarbamoyl) Cysteine (AMCC). A reactive intermediate (between NMF and AMCC), namely Methyl Isocyanate (MIC), is formed and reacts with glutathione to form its S-conjugate metabolite, which probably acts as a toxic metabolite [36]. Furthermore, adducts can be formed to globins via the NMHB (N-Methylcarbamoyl) group that binds to the N-terminal valine [37].

Consecutive to its metabolism, DMF generates various electrophilic metabolites which avidly consume glutathione and bind to nucleophilic sites of intracellular macromolecules. The free radicals released by the action of CYP2E1 on high doses of DMF can attack cell proteins and inactivate the P450 cytochrome [38].

Besides the metabolites formed from DMF, some endogenous metabolites were also identified by the PLS-DA (summary in table 1) as discriminating descriptors. Most of them were found in lower
concentrations in the urine of DMF-exposed animals compared to pre-test samples. Among them, hippurate was mostly affected due to liver damage (cf. VPA). Glucosuria was also decreased in DMF-exposed rats, most likely as a consequence of the hypoglycemia observed during the early stage of steatosis [39]. Combined with the reduction in the urine levels of several Krebs cycle intermediates, this demonstrates a significant lack in cellular energy (citrate, α-ketoglutarate, and succinate). The lack of glucose forcing the neoglucogenesis from glycogen was previously reported by Abdul Mutlib et al., who demonstrated an upregulation of Udpg2 (UDP-glucose pyrophosphorylase), an enzyme involved in the biosynthesis of glycogen. The same authors also evidenced the steatogenic phenomenon by the upregulation of HMGCGR (increased cholesterol) and a downregulation of LIPG (normally transforming TG into AG) [40]. These two enzymatic changes are crucial to the toxic mode of action of DMF. The drop in glutathione was evidenced in this study by the reduction in dimethylglycine and betaine, which are both involved in the formation of the antioxidant molecule.

Conclusions

Although the mechanisms of action of the three steatogenic molecules used in this study are different, they all lead to an alteration in the Krebs cycle activity due to a lack of acetyl-CoA. The energy deficit produced dropped very quickly, as 4 hours after exposure a decrease in the concentrations of various Krebs cycle intermediates was already noticed in the urine of exposed animals.

Decreased hippurate urine level was also seen in animals receiving any of the steatogenic compounds. This effect is due to the drop in coenzyme A used for the conjugation of benzoate and glycine to form hippurate. Moreover, this ATP-dependent reaction suffers from the lack of energy already mentioned. The body favors the use of CoA in producing energy instead of hippurate, despite the risk of limiting the removal of nitrogenous waste.

Moreover, even if glycine was not visible in the proton NMR spectra of urine samples due to overlaps in its resonances with those of sugars, it is clear that the biochemical pathways in which it is involved were altered. Indeed, reductions in the levels of guanidoacetate, creatine and creatinine were also observed for two of the tested drugs. This lack in glycine further compromises the synthesis of hippurate and above all, of glutathione, the major detoxifying molecule.

Steatosis induced by a xenobiotic, with adverse and irreversible effects on the body, is a slow process and requires repeated drug exposure. The aim of this work was to identify a urine metabolomic signature of the early events following the inhibition of β-ox in rats. The exclusive analysis of a urine samples does not necessarily reflect the entirety of the metabolic disturbances induced by a xenobiotic. Therefore, biomarkers directly related to onset of steatosis did not emerge consistently for the three tested molecules. Nevertheless, the inhibition of β-ox caused a severe energy crisis in rats within 24h of exposure to any of the tested steatogenic molecules. This energy crisis was the starting point of more specific alterations in other metabolic pathways. These later changes are currently under investigation in refined experimental protocols, including the isolated and perfused liver model and human hepatocytes cell cultures. The results are encouraging and may provide better understanding of the biochemical pathways involved in DILI. Indeed, excreted metabolites involved in the urine metabolomic signature identified in rats could also be observed in human urine and share common biochemical and cell signaling pathways.

In conclusion, a metabolomic pattern reflecting an inhibition of the β-ox pathway emerged as an early cellular alteration eventually leading to drug-induced hepatic steatosis. Such a metabolomic evaluation could be applied to other metabolic disorders consecutive to a mitochondrial dysfunction.

Methods

Chemicals: N, N-Dimethylformamide (DMF, D/3841/08) was purchased from Fisher Chemicals (Fisher Scientific, Aalst, Belgium). 3-(Trimethylsilyl) Propionic-2,3,3-d4 acid (TSP), D3-O, Valproic Acid Sodium salt (VPA, P4543) and Tetracycline hydrochloride (TC, T3383) were purchased from Sigma (Sigma, Diegem, Belgium). Test solutions of each of these compounds were prepared in sterile saline for i.p. injections.

Animals and drug treatments: All experiments were approved by the Ethical Committee of the Institution. Male Wistar Han rats weighing 200 - 300 g at the onset of the study were individually housed in metabolism cages with free access to food and water. Animals were maintained under a 12 h light/dark cycle at 22 ± 1°C and a humidity level of 60%. After a 2-Day period of acclimating, animals were intraperitoneally exposed to VPA (750mg/kg, n=6), TC (250mg/kg, n=6) or DMF (1000mg/kg, n=6). Doses were selected based on a thorough review of the literature showing induction of steatosis in acutely exposed rats [41-43]. Drugs were injected i.p. to anesthetized animals (anesthesia was induced with isoflurane 4% at a flow rate of 1.0 l/min, and maintained with isoflurane 1.5 % at a flow rate of 0.5 l/min). The day before dosing, pre-test urine samples were collected. After dosing, urine fractions were collected every 4 hours for 24h in refrigerated tubes containing 125μl of sodium azide 1.0%.

Sample preparation and 1H-NMR spectroscopy analysis: Urine samples were centrifuged at 1600 g for 5min at 4°C. Then, 400μl of urine was mixed with 200μl of phosphate buffer (0.2 M Na2HPO4/0.04 M NaH2PO4, pH 7.4) and prepared in a mixture of H2O/D2O (80:20; v:v), to minimize pH variation. Then the samples were centrifuged at 13,000 g for 10 min. 50μl of a 12 mM solution of 3-(Trimethylsilyl) Propionic-2,2,3,3-d4 acid (TSP) solution prepared in 100% deuterium oxide was added to 550μl of the supernatant.

600μl of this mixture was then transferred into 5-mm NMR tubes and 1H-NMR spectra were recorded at 302 K using a Bruker Avance 400 spectrometer working at 9.4T (400MHz proton Larmor frequency). One-dimensional spectroscopy was performed using a NOE-SYPRESAT-1D pulse sequence, 64 scans, 32,768 data points, a spectral width of 6410.2 Hz, an acquisition time of 2.5 s and a pulse recycle delay of 2 s. FIDs were Fourier transformed and a line broadening of 0.3 Hz was applied. The spectra were automatically phase- and baseline-corrected using MestReNova 5.2.0 software (Mestrelab Research, Santiago de Compostela, Spain). Spectral chemical shifts were calibrated according to the resonance of TSP, arbitrarily fixed at 0.00 ppm and all peak intensities were normalized to the reference, whose intensity was arbitrarily fixed at a value of 100.

Multivariate data analysis: The spectral region ranging from 0.08 to 10.00 ppm was reduced to 0.04 ppm buckets to obtain 248 sub-regions. The regions ranging from 4.50 to 5.18 ppm and from 5.50 to 6.18 ppm were excluded from the analysis to remove the residual water signal and the contribution of the urea resonance that may fluctuate due to daily variations. When present, the resonances arising from the model substances and/or their metabolites were also excluded before further analysis of the spectra. Each integrated...
region was normalized to the total spectrum area to compensate for possible variations in urine dilution/concentration effects among the samples. The final data set was imported into SIMCA-P+ 12.0 software (Umetrics, Umeå, Sweden). After mean-centering without scaling, the dataset was firstly investigated by an unsupervised Principal Components Analysis (PCA) in order to highlight any grouping or separation between the data. It has to be stressed that pre-test samples were compared to urine collected from control animals and showed remarkably similar metabolic compositions.

In a second step, Projection to Latent Structure Discriminant Analysis (PLS-DA) was applied to the same dataset in order to identify which spectral sub-region(s) and consequently which urine metabolites, were responsible for potential differences between drug-exposed and pre-test animals. The effects caused by each tested compound were evaluated by measuring the changes occurring in urine samples collected at different time points after dosing as compared to pre-test samples, collected during the 24h period preceding dosing. For the dynamic evaluation of the metabolic changes, samples were grouped as follows: from 4 to 8h, 12 to 16h and 20 to 24h post-dose.

Selection of discriminant descriptors and confirmation of metabolite changes: The selection of data was based on the VIP criterion (Variable Importance in Projection) [44]. In this study, the variables were considered as representative when their VIP was above 1. Variables with a VIP ≥ 0.8 were also taken into account, but should be interpreted as a trend rather than a significant effect. Metabolites responsible for any segregation between the exposed and pre-test animals were identified from the chemical shifts and multiplicities of their corresponding resonances. Identifications were made possible by matching the hypothetical structures with databases available on the web (such as Human Metabolome Data-Base, HMDB) and by comparison with in-house reference tables.

Statistical significance was determined by integrating the NMR peaks of each of the metabolites that contribute to the multivariate separation using a Wilcoxon matched-pairs signed-ranks test and significance was determined at P < 0.05 (*) and P < 0.01 (**).

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