METABONOMIC FINGERPRINT OF THE ISOLATED AND PERFUSED LIVER.

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Background :

Metabonomics studies based on spectroscopic analysis of biofluids are a useful tool to explore the biological and metabolic profiles of an organism. However, biofluids are a mixture of numerous metabolites with various tissue origins, making it very difficult to determine which organ/tissue a particular metabolite is arising from (fig.1).
This is particularly troublesome when attempting to validate urinary markers of hepatotoxicity because most of them could as well be excreted by extra hepatic cells. In order to overcome this issue, we applied the metabonomic approach to the **isolated and perfused rat liver** (IPRL).

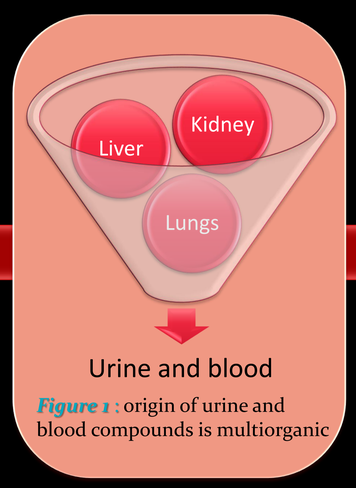


Figure 1 : origin of urine and blood compounds is multiorganic

The IPRL is a valuable ***ex vivo* model** commonly used in metabolic, transport, PK/PD, and toxicology evaluation of xenobiotics.
Indeed, the liver tissue can be exposed to the test substance independently from other organs. The perfusion fluid can then be sampled over time and subsequently analyzed by spectroscopic techniques (NMR or SM) for metabolic as well as risk assessment purposes. Finally, as compared to cultured hepatocytes, the IPRL model maintains the tissue architecture, cell polarity and bile flow.

Applying the metabonomic approach to the IPRL model should allow us to undoubtfully **identify** which markers are indeed of hepatic origin.

Material and methods :

Wistar male rats placed in metabolic cages (fig. 2) received either a single dose of **hydrazine** (100 mg/kg, i.p.) or 2 doses of **valproic acid** (750 mg/kg, s.c.). **Urine** samples collected before and after dosing were analyzed **by** **^{1}H-NMR** spectroscopy at 400 MHz.
After processing and binning, multivariate data analysis was applied to the NMR data (Umetrics, SIMCA-P+).

Figure 2. **metabolism cage** with wistar rat.

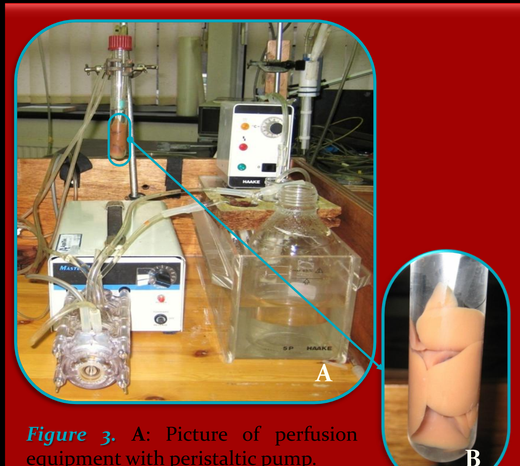


Figure 3. A: Picture of perfusion equipment with peristaltic pump.
B: Isolated liver.

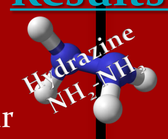
Livers isolated (fig.3b) from male Wistar rats were perfused through the portal vein with a recycling Krebs-Henseleit solution using a peristaltic pump (fig.3a). Hydrazine or valproic acid were added to the fluid (same doses as used *in vivo*). Perfusion fluid was sampled every 15 minutes for two hours. Samples were lyophilized and prepared for ^{1}H-NMR spectroscopy. During the entire experiment, liver viability was assessed by ^{31}P NMR and bile flux (>1µl/min/g liver weight). Liver resections were prepared for histopathology review.

Results :

	Metabolite	Toxicity
↑	Creatine	Liver toxicity
↑	Taurine	Steatosis / osmoprotection
↑	N-acetylcitrulline + Citrulline	Inhibition urea cycle
↑	2-aminoadipate	Neurologic toxicity
↓	Citrate	Krebs cycle intermediates → mitochondrial toxicity
↓	Succinate	
↓	α-ketoglutarate	

Table 1 : increase and decrease of metabolites in urine of rat treated with hydrazine

As already reported by others, our *in vivo* investigations confirmed the increased excretions of taurine and creatine in urine of rats treated with hydrazine and histological figures confirmed the accumulation of lipids (steatosis) within the midzonal region of the liver.



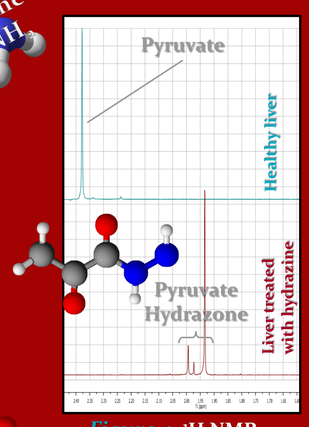
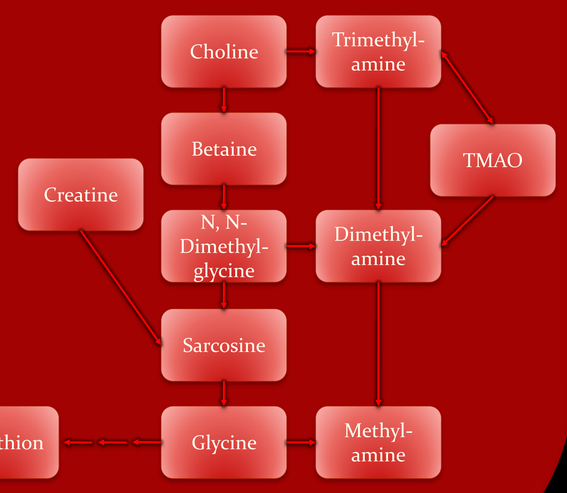


Figure 4. **^{1}H NMR spectra, at 500MHz,** of perfusion media.

When hydrazine is added to the fluid, it spontaneously **complexed** with pyruvate to form **pyruvate hydrazone** (fig. 4). Hence, the availability of pyruvate for hepatocytes was dramatically reduced, causing a deprivation in energy stores. Furthermore, bound hydrazine was unable to exert its hepatotoxicant role. The unavailability of pyruvate forced liver cells to switch to other biological pathways to compensate : biomarkers of protein catabolism (amino acids), β-oxidation and ketone bodies (α and β-hydroxybutyrate) were found at higher urinary levels. In addition, histological data showed large decrease in liver glycogen content (most likely used for glycogenolysis).

	Metabolite	Toxicity
↑	Adipate	Lipid accumulation-steatosis
↑	B–hydroxybutyrate	Carbohydrate metabolism
↓ 8 and 16 hours after injection	Taurine	Osmoprotection
↓	Creatine	Glutathione depletion
↓	Betaine	
↓	Dimethylglycine	
↓	Sarcosine	
↓	Krebs cycle intermediates	Mitochondrial toxicity

Table 2 : increase and decrease of metabolites in urine of rat treated with valproate.



VPA interferes with branched amino acids (aas) and fatty acid metabolisms. VPA inhibits β-oxydation, causes steatosis, and induces depletion of glutathione. Moreover, an alteration of the methionine metabolism was also caused by VPA.

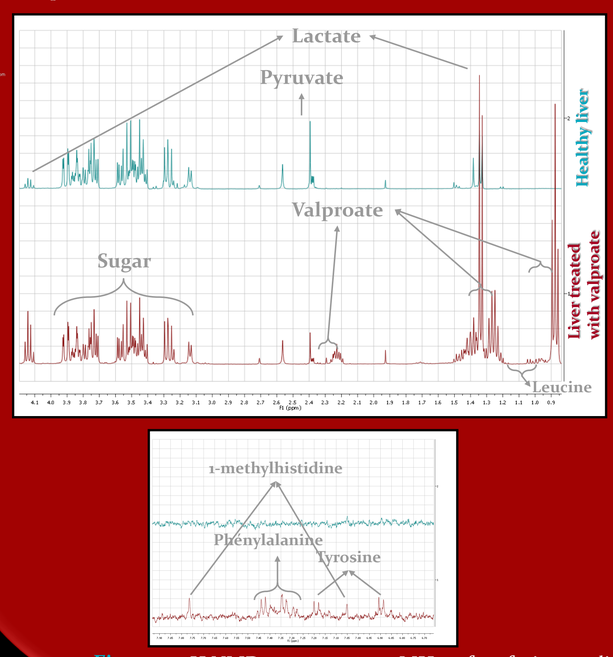


Figure 5. **^{1}H NMR spectra, at 500MHz,** of perfusion media.

When healthy livers were exposed to VPA via the perfusion fluid, specific changes in the ^{1}H-NMR spectra were observed as compared to pretreatment fingerprint (fig. 5). However, these changes did not correspond exactly to those observed in vivo. One major difference was the large amount of aas released in the perfusion fluid during exposition of the livers to VPA.

Perspectives:

Discovery of new biomarkers of drug-induced toxicity is a promising application of metabonomics. However, urinary or plasma components may originate from many different tissue/organs which makes it very difficult to validate such markers. The isolated and perfused organ approach could be a very useful tool to **confirm the tissue origin of proposed biomarkers before their validation**. However, a thorough control of the experimental conditions, such as close or open circulation, presence or absence of albumin in the perfusion fluid,... is necessary to avoid false interpretation.