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Research Paper

IDENTIFY BIOMARKERS INVESTIGATION IN HEPATIC DYSFUNCTION IN CCl_4 INDUCED LIVER DAMAGE IN RATS

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Identify Biomarker Investigation in Hepatic dysfunction in CCl_4induced Liver damage in Rats. CCl_4 was the chosen hepatotoxicant since it has been widely used in rat models for liver injury. Extensive 1D 1H NMR-based metabolomics investigations were carried out to determine changes in urinary levels of metabolites with acute and chronic hepatic injury. Urine was chosen as the preferred biofluid since it can be obtained using non-invasive methods. 1D 1H NMR combined with PCA/OPLS analysis of the urine samples collected at different ages, also revealed significant differences to the concentration of urinary metabolites. There was a general trend of a dramatic increase in urinary levels of many metabolites at 84 days followed by a decrease at 91 days. Metabolites which followed this pattern included creatinine, creatine, taurine, 2-oxoglutarate, citrate and succinate. This proved that the urinary metabolites were affected by the male sexual maturation process. Using the information collected from our acute liver injury model we developed a model of CCl_4-induced fibrosis in the rat. Our model involved the administration of 0.4 ml/kg CCl_4, 3 times a week for 6 weeks. We included a 6 week period without treatment at the end of the dosing period to evaluate the potential of reversibility of the lesion.

Key Words: Creatinine,Hepatotoxicant,PCA/OPLS analysis.

INTRODUCTION

Liver, the largest organ in the vertebrate body - is the major site of intense metabolic activities such as drug and xenobiotic metabolism. Liver injury caused by toxic chemicals and certain drugs has been recognized as a crucial toxicological problem. In the present world a large number of toxins are introduced daily. So it is more important than ever to keep the liver healthy and potent. The most important metabolic function of liver is the detoxification and excretion of toxic chemicals, drugs and hormones. Liver tissue has the capacity to regenerate, so a moderate cell injury is not reflected by measurable change in its metabolic function. Due to the high tolerance of liver, liver disease is seldom detected at the early stage and once detected treatment faces a poor prognosis in most cases. Plants have formed the basis of sophisticated traditional medicine (TM) practices that have been used for thousands of years by people in China, India, and many other countries. Some of the earliest records of the usage of plants as drugs are found in the Artharvaveda, which is the basis for ayurvedic medicine in India. Ayurveda has a long tradition in treating various diseases including liver diseases using herbal medicines. Apart from timely cure the ayurvedic herbs give a permanent relief from the diseases by removing the metabolic toxins from our body.

Aim and Objective: The aim of the current studies is to identify Protein andBiomarker Investigation in Hepatic dysfunction in CCl_4induced Liver damage

in Rats. Urine was chosen for biomarker identification since it can be easily collected in relatively large volumes and without any associated pain, distress and discomfort to the animal. The identification of non-invasive markers of liver damage that could be used in the preclinical toxicology assessment and clinical safety testing of drugs would provide relevant information to the process of drug development and could be extremely useful in monitoring target organ toxicity in clinical trials.

In the present investigations animal models of liver toxicity will be developed using CCl_4 as the hepatotoxicant. Initially, a single administration of CCl_4 at a range of concentrations will be used to determine the optimal dose level for our acute hepatic injury model. CCl_4 exerts its toxic effects mainly in the liver; however, injury has also been detected in other organs, including the kidneys. Therefore, our primary goal will be to identify the dose level of CCl_4 that is below the threshold for nephrotoxicity. To reliably determine the presence of renal damage we will investigate the sensitivity of a panel of kidney injury biomarkers recently approved by the FDA to use in toxicology studies. These will be used in our hepatic injury model to confirm the absence of a CCl_4induced toxic effect in the kidneys. Confirmation of absence of renal damage will be made by means of histopathological examination of kidney samples collected at autopsy.

With the preliminary information collected from the dose response study regarding the most sensitive urinary markers of renal damage we will then focus on the development of a model of CCl_4 induced liver fibrosis in the male rat.

- Biomarker identification. In this situation, it would be difficult to determine if biomarkers identified in our study were present as a result of hepatic toxicity, nephrotoxicity or both. Consequently, in this study, great importance was attached to finding a dose level that induced hepatotoxicity but not nephrotoxicity.
- Serum samples obtained from animals in the study were analysed for serum enzymes. Levels of hepatic leakage enzymes such as ALT, AST and GLDH, are described as markers of hepatic injury and could thus help to confirm injury.

1H Nuclear Magnetic Resonance (NMR) spectroscopy

Urine samples for 1H NMR spectroscopy were prepared by the addition of 200 µL buffer solution (0.2 M Na_2HPO_4/0.2 M NaH_2PO_4, pH 7.4) to 400 µL of urine. The mixture was allowed to stand at room temperature for 10 minutes followed by centrifugation at 13,000 rpm for 10 minutes. 500 µL aliquots were placed in 5 mm NMR tubes to which 50 µL of a standard solution of TSP in D2O was added (final concentration = 1 mM). The D_2O and TSP provide both a chemical shift reference (δ 0.0) and a deuterium lock signal for the NMR spectrometer.[31]

One-dimensional 1H NMR spectra of urine were measured at 500.00 MHz on a Bruker DRX-500

spectrometer using a standard pre-saturation pulse sequence for water suppression with irradiation at the water frequency during the relaxation delay of 3 s and the pulse sequence mixing time of 100 ms. Following 4 dummy scans, spectra were acquired using 64 scans into 64K points using a spectral width of 10,080 Hz, an acquisition time of 4.68 s, and a total pulse recycle time of 7.68 s. The free induction decays (FIDs) were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz prior to FT.[32,33]

All 1H NMR spectra were phase and baseline corrected using TopSpinRheinstetten, and data reduced using AMIX to regions 0.04 ppm wide from δ 10.0 to 0.2 producing 250 chemical shifts (variables), an operation known as binning or bucketing. The bucket size was chosen to allow for small changes in chemical shift due to variations in pH within the samples. The region between δ 6.1-4.5 in the urine spectra was set to zero integral value for the purposes of pattern recognition analysis to remove the variability in presaturation of the water resonance and cross-relaxation effects on the urea signal.

The second step in data processing is a row operation known as normalisation. For each region of the spectra (bucket) (minus the water region), the area under the curve was calculated and expressed as an integral value. All regions of the spectra were normalisedto the sum of the integrals to reduce any significant differences in concentration between individual urine samples. The data were then imported into Microsoft Excel and converted into a bucket table. Each bucket table was then imported into SIMCA 13. The pre-processed data was compiled to create a bucket table where the rows represent individual samples and the columns (comprising 250 variables/buckets) represent integrated and normalised peak areas.[34,35,36]

Finally, a step is carried out to reduce the noise in the data. This is a column operation that acts on each spectral intensity across all samples, a process known as scaling. Mean-centering scaling was carried out in which the column mean is subtracted from each value in the column, so that each column has a mean of zero. This was followed by Pareto scaling where each variable is divided by the square root of its standard deviation.[37]

Pattern recognition analysis of 1H NMR spectral data of urine and serum

PCA, OPLS and OPLS-DA were performed using SIMCA 13. PCA is an unsupervised method which is used to identify similarities and differences between samples from controls and treated animals. It reduces the dimensionality of a data set by creating a new set of variables, known as Principal Components (PCs). PCs are uncorrelated, with the first component (PC1) explaining the largest variance in the data and the subsequent PCs describing progressively smaller proportions of the total variance. PCA was

performed to determine the presence of patterns and outliers among samples which appeared distinct from other samples. Outliers are shown outside the Hotelling's T2 elipse which defines the 95 % confidence interval. In the present studies, outlier samples that fell outside the Hotelling's T2 plot were individually analysed and excluded from further analysis when necessary.

The PCA scores plots were used to identify the presence of inherent clustering patterns between groups of samples and the corresponding loadings plot to identify the regions in the spectra that most contributed to the separation of groups of samples on the scores plot.

OPLS is a regression model used to describe the relationship between a descriptor matrix X, and a response matrix Y containing quantitative values. When OPLS is applied to qualitative data, such as discriminant analysis it is known as OPLS-DA. A variable importance plot (VIP) was also used to identify putative markers of CCl_4-induced toxicity. This type of plot describes which X variables characterise the X matrix well and which variables correlate with Y. The VIP values summarise the overall contribution of each X-variable to the OPLS model. Variables with small VIP scores, usually less than 0.5, are less important and can therefore be excluded from the model.

Variables identified from the VIP plot as contributing to sample separation were then matched to chemical shifts in the 1H NMR spectra. Identification was carried out by visual comparison of peaks in the 1H NMR collected from this project with peaks in spectra from previously published literature.

The quality of the PCA, OPLS and OPLS-DA models was calculated by determining the predictive ability of the models (Q2) and the correlation coefficient (R^2) which is a measurement of how well the model fits the data. The predictive ability (Q2) was evaluated by performing a cross-validation (CV). Q2 values greater than 0.5 are generally considered good.The greater the value for R2 the better the fit.

Statistical analysis

Urine data were corrected for volume and are reported as 'per collection period' (c.p.). Urine, and serum clinical chemistry data were log transformed before analysis. Data are presented as means (SD) for groups of animals.

Group means and SD were compared by one-way analysis of variance (ANOVA) followed by a post-hoc Dunnet's test, or by the means of a Student's t-test using the software package SPSS Significance was set at *P<0.05, **P<0.01 and ***P<0.001.

- For 1H NMR statistical analysis of integral regions, a Student's t-test was performed.

Dose response study for the identification of a dose level to induce hepatotoxicity but not injury to other organs following CCl_4 administration

In the present studies carbon tetrachloride (CCl_4) was chosen as the hepatotoxicant agent since it is one of the most commonly used compounds for the investigation of liver toxicity. CCl_4 is metabolised in

the liver by cytochrome P-450 2E1 to produce reactive free radicals. Since the concentration of CYP450 is greatest in the centrilobular region of the liver this area is most susceptible to injury. However, CCl_4 is highly lipophilic and therefore can be readily distributed throughout the body resulting in injury to other organs such as the kidneys, the lungs and the brain.

1H NMR spectrometry

Urine samples were collected from rats between 6 and 24 hours post-dosing with a single dose of CCl4 at 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2 and 3.6 mL/kg. Urine samples were prepared by the addition of D2O and analysed by 1D 1H NMR to assess changes in the urinary metabolite profile.

Visual inspection of characteristic 1D 1H NMR spectra of urine samples revealed similarities between the metabolite profiles of CCl4-treated animals and control rats. However, some differences (e.g. change in peak intensity/concentration) in the profiles were evident particularly when urine from 3.6 mL/kg CCl4-treated animals (highest dose group) was compared to controls. Peaks displaying changes due to CCl4-induced injury included taurine (δ 3.14, δ 3.30), succinate (δ 2.30), creatine (δ 2.94, δ 3.78), creatinine (δ 3.02), 2-oxoglutarate (δ 2.34, δ 2.90), hippurate (δ 3.86, δ 7.54, δ 7.58, δ 7.74), fumarate (δ 6.54) and citrate (δ 2.42, δ 2.62).

Before analysis, spectra were pre-processed and multivariate data analysis was carried out using mean-centered data and Pareto-scaling as described in Section 2.11. PCA, an unsupervised pattern recognition method was used to analyse the spectra from the 6 animals at each dose level. PCA is a pattern recognition technique that allows separation of samples into clusters according to the similarities between them. Results can be visualized into two types of plots, the scores plot where each point represents an individual sample, and allows the observation of patterns, clusters and outliers; and the loadings plot, where each point represents a variable, i.e., a spectral signal. The loadings plot provides the basis for metabolite differences interpretation and identification. The PCA scores plot was obtained from the 1D 1H NMR analysis of the urine samples. There was no evidence of distinct clustering patterns between control and CCl4-treated samples at the different dose levels and many of the data points displayed overlap (Figure 3.6). Outlier samples that fell outside the Hotelling's T2 plot were individually analysed and excluded from further analysis when necessary.

The lack of distinct clustering patterns between the control and CCl4-treated samples may have been due to the type of data analysis (PCA) carried out. Therefore, to further investigate the possibility of metabolomic differences between vehicle-treated (control) and CCl4-treated samples, a supervised pattern recognition method (OPLS) was used. OPLS is a prediction and regression method that finds the information in the X data (which can be 1D 1H NMR spectral data) that is related to known information, the Y data (which can be class information, treatments, time or dose).

The OPLS scores plot of urine samples treated at 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2 and 3.6 mL/kg CCl4. There was an increased degree of sample separation compared to the PCA model (Figure 3.6), and it appeared there was a dose-related trend. As the CCl4 dose level increased there was a shift towards the right hand side of the plot. Consequently, urine samples from control and CCl4 treated groups at 0.4, 0.8, 1.2 and 1.6 mL/kg were mainly located on the left half of the plot, whereas urine samples from higher CCl4 dose levels (2.0 mL/kg CCl4 and above) clustered on the right half of the scores plot.

A second OPLS model was constructed after exclusion of urine samples from animals treated with CCl4 at dose levels above 2.0 mL/kg. At these higher dose levels, histopathological examination confirmed CCl4-induced kidney injury. Therefore, metabolite changes in the urine from animals dosed with CCl4 above 2.0 mL/kg may reflect nephrotoxicity as well as hepatotoxicity. Since the aim of these studies was to identify biomarkers of hepatotoxicity, the elimination of these samples from the analysis ensures that changes in metabolite levels are solely due to liver injury.

The scores plot and the corresponding loadings plot for the OPLS model. The scores plot shows the presence of 6 different clusters corresponding to each of the treatment groups (controls, 0.4, 0.8 1.2, 1.6 and 2.0 mL/kg). A clear dose-dependent separation along the first component (t[1]) is present; arrows in the scores plot represent the shift along PC1. By removing CCl4-treatment groups where urinary renal toxicity-specific metabolites may have been present we were able to eliminate confounding factors that were unrelated to the effect studied and therefore enhance the correlation between the X matrix and the Y variable of the OPLS model.

The loadings plot reveals the chemical shifts which contribute to sample separation in the scores plot. The position of each NMR variable on the loadings plot is related to the direction of its contribution to the separation of samples on the scores plot. Therefore, spectral regions corresponding to the chemical shifts located on the right hand side of the loadings plot were increased in urine samples from CCl4-treated rats at the higher dose levels.

To further investigate the differences between control and treated samples an OPLS-DA model was used. When the Y variable in an OPLS model is a discrete variable (for example, male or female, control and treated or different dose levels, like in the present study), the model becomes an OPLS-DA model. OPLS-DA models are mostly useful in sample discrimination, that is, in the identification and classification of potential biomarker candidates and in the separation of multiple treatments (Rezzi et al., 2007). In the OPLS-DA model, control samples were treated as class 1 and CCl4-treated urine samples were treated as class 2. Consequently, the data was modelled so that information contributing to sample separation was forced onto the first component (t[1]), whereas orthogonal information relating to intra group

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variability was modelled in the successive components.

Interpretation of an OPLS-DA loadings plot is difficult to achieve with more than 2 classes due to the presence of multiple sample clusters which all have the ability to influence the loadings plot simultaneously. Therefore, individual OPLS-DA models for control and treated samples at 0.4, 0.8, 1.2 and 1.6 mL/kg CCl4 were performed and are shown in Figure 3.9. These plots revealed a very good degree of separation between control and treated samples along the discriminating component t[1]. In all plots, control samples clustered on the left hand side of the scores plot and CCl4-treated samples clustered on the right hand side.

In this study, the maximum CCl4 dose level which induced hepatotoxicity without causing kidney injury was 2.0 mL/kg as confirmed by histopathological examination which reported the absence of a CCl4-induced nephrotoxic effect at this dose level. Therefore, this dose level was chosen as the optimal CCl4 concentration for the further investigation of potential biomarkers of hepatotoxicity.

The OPLS-DA scores plot, corresponding loadings plot, S-plot and VIP plot for control samples and samples treated at 2.0 mL/kg CCl4 are shown in Figure 3.10. The S-plot is used to visualise the OPLS-DA loadings plot and helps to extract potential biomarkers. An ideal biomarker should have a high magnitude and high reliability; therefore, variables which are responsible for most of the clustering separation will be located at the extremes of the S-plot. The upper right quadrant of the S-plot shows the chemical shifts that are increased in CCl4-treated samples, whereas chemical shifts located in the lower left quadrant of the S-plot are increased in control samples.

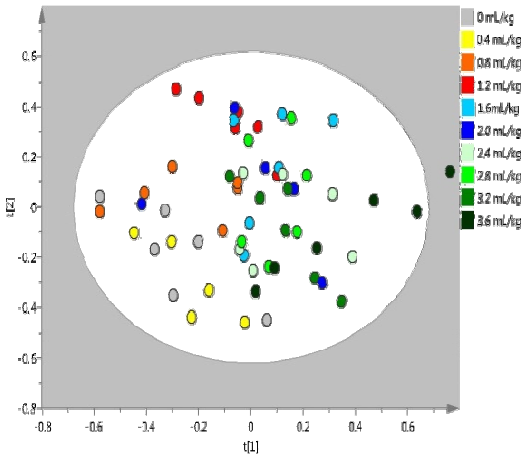

Fig. 1: PCA scores plot from a PCA model derived from 1D ^{1}H NMR spectral data of urine samples from male Hanover-Wistar rats treated with increasing doses of CCl_4.

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International Journal of Pharmaceutical Erudition

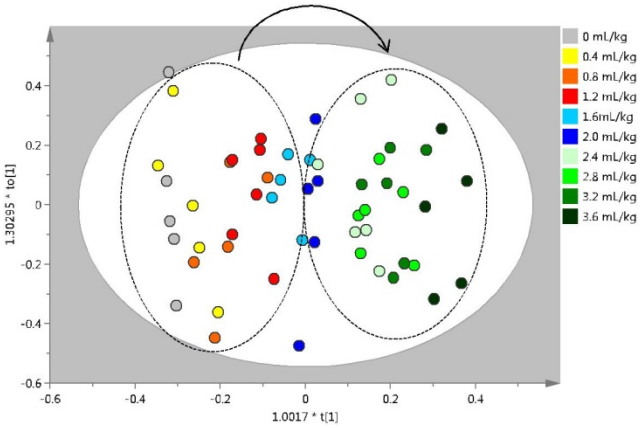

Fig.2: OPLS scores plot from an OPLS model derived from 1D ^{1}H NMR spectral data of urine samples from male Hanover-Wistar rats treated with increasing doses of CCl_4.

A second OPLS model was constructed after exclusion of urine samples from animals treated with CCl_4 at dose levels above 2.0 mL/kg (Figure 3.8). At these higher dose levels, histopathological examination confirmed CCl_4-induced kidney injury. Therefore, metabolite changes in the urine from animals dosed with CCl_4 above 2.0 mL/kg may reflect nephrotoxicity as well as hepatotoxicity. Since the aim of these studies was to identify biomarkers of hepatotoxicity, the elimination of these samples from the analysis ensures that changes in metabolite levels are solely due to liver injury.

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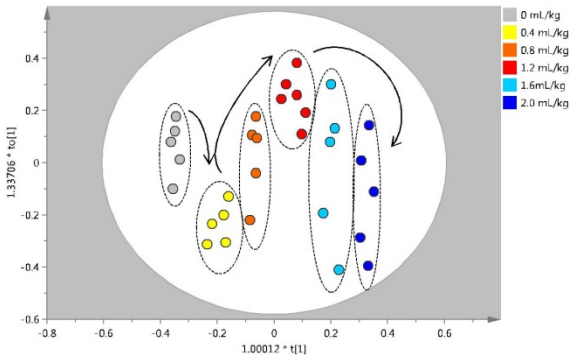
(A)



(B)

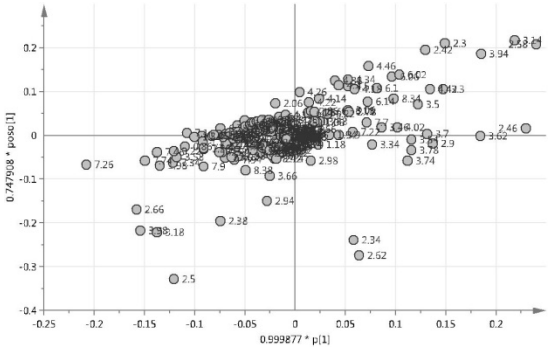


Figure 3: OPLS scores plot (A) and loadings plot (B) from an OPLS model derived from 1D ^{1}H NMR spectral data of urine samples from male Hanover-Wistar rats treated with increasing doses of CCl_4.

Interpretation of an OPLS-DA loadings plot is difficult to achieve with more than 2 classes due to the presence of multiple sample clusters which all have the ability to influence the loadings plot simultaneously. Therefore, individual OPLS-DA models for control and treated samples at 0.4, 0.8, 1.2 and 1.6 mL/kg CCl_4 were performed.These plots revealed a very good degree of separation between control and treated samples along the discriminating component t[1]. In all plots, control samples clustered on the left hand side of the scores plot and CCl_4-treated samples clustered on the right hand side.

In this study, the maximum CCl_4 dose level which induced hepatotoxicity without causing kidney injury was 2.0 mL/kg as confirmed by histopathological examination which reported the absence of a CCl_4-induced nephrotoxic effect at this dose level. Therefore, this dose level was chosen as the optimal CCl_4 concentration for the further investigation of potential biomarkers of hepatotoxicity.

The OPLS-DA scores plot, corresponding loadings plot, S-plot and VIP plot for control samples and samples treated at 2.0 mL/kg CCl_4. Variables of interest were chosen based on the variable importance in the projection plot (VIP) values (VIP≥1), having a high p[1] value and p(corr)[1] value. Each of these variables represents a chemical shift region (bin). Therefore, the NMR spectra were visually inspected at each of the regions identified. Peaks in these regions were evaluated for chemical shift value and multiplicity and this data was compared with data in previously published literature. Several other chemical shifts were also identified as major contributors to class separation but could not be assigned to metabolites. Subsequently, a list of some of the metabolites showing changes between control and 2.0 mL/kg CCl_4-treated samples was constructed. A Student's t-test was carried out to determine the statistical significance of the difference in the integral values for the chemical shift regions.

The most relevant changes in the 1D ^1H NMR urinary metabolomic profile of male Hanover-Wistar following the administration of 2.0 mL/kg CCl_4 include an increase in the resonances of taurine, formate, creatine, 2-oxoglutarate, citrate, glucose and succinate, and a decrease in the resonances of hippurate, fumarate and creatinine. These changes in resonance relate to increases and decreases in the urinary concentration of these metabolites.

Dose response study for the identification of a dose level to induce hepatotoxicity but not injury to other organs following CCl_4 administration

Observations during the study

Throughout the study, animals were observed for signs of ill-health and clinical observations were recorded during the post-mortem procedure. The behaviour of the animals was observed to guarantee their welfare and as a first indicator of pain and distress. During the period in the metabolism cages, animals treated with CCl_4 at

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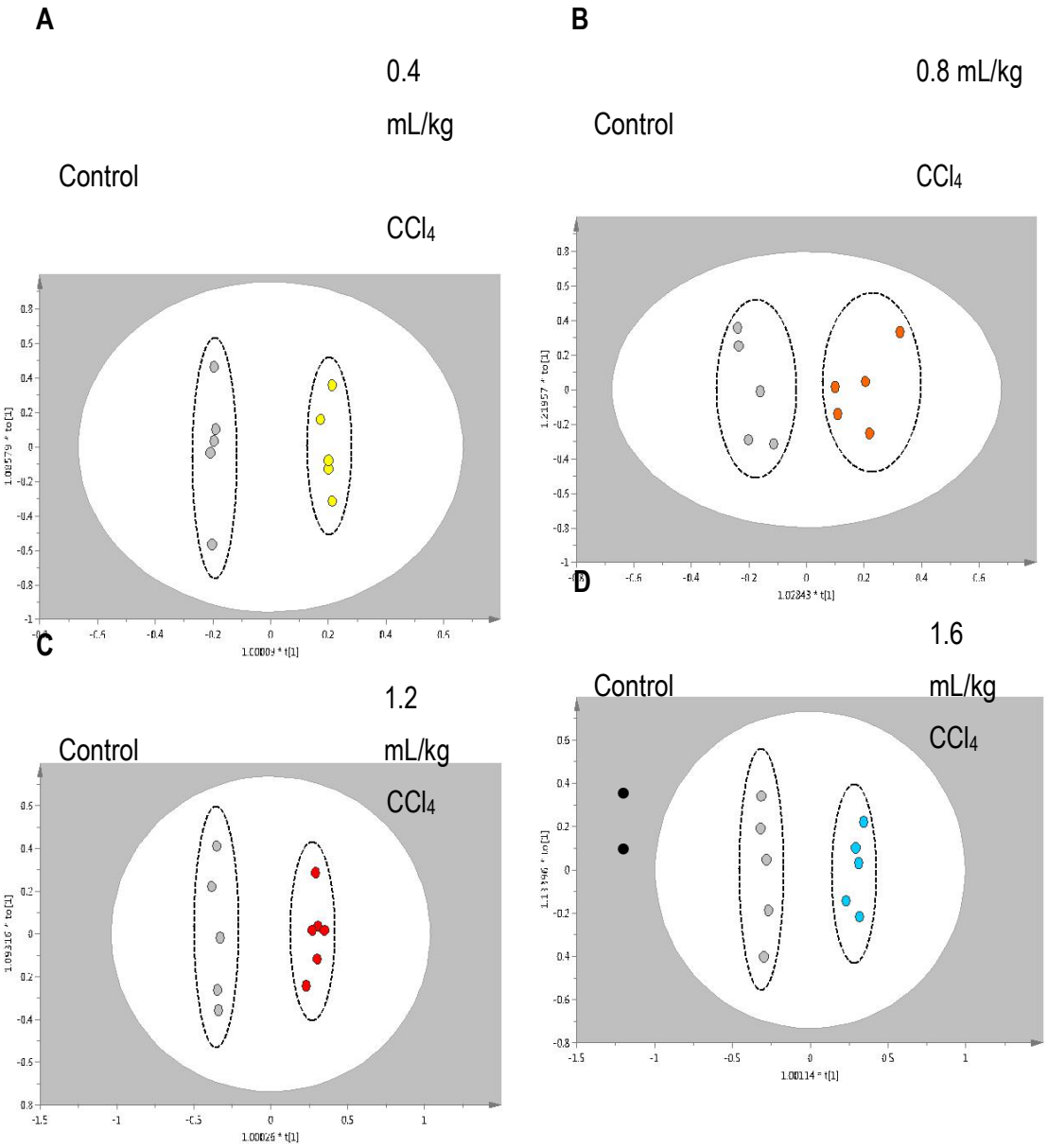
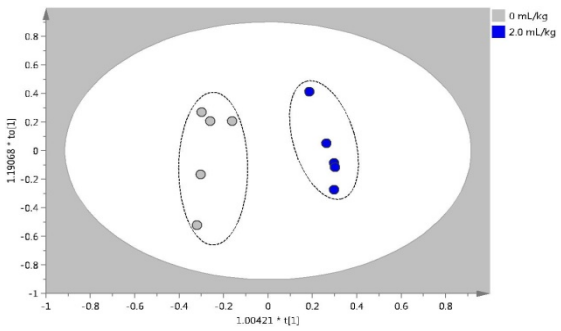


Figure 5.7 Scores plots from OPLS-DA models derived from 1D ^{1}H NMR spectral data of urine samples from male Hanover-Wistar rats treated with increasing doses of CCl_4.

(A)



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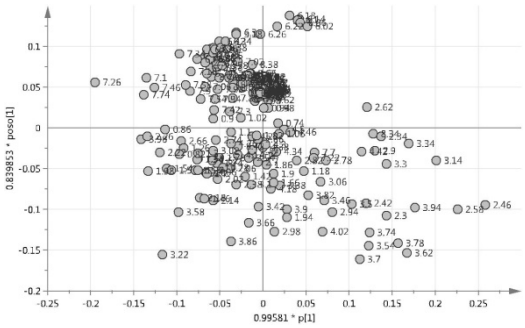

(C)

(D)

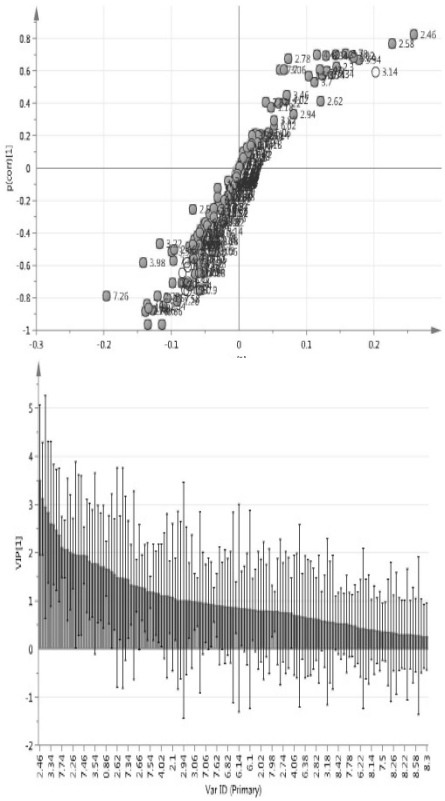

Fig. 4: 8 Scores plot (A), loadings plot (B), S-plot (C) and VIP-plot (D) from an OPLS-DA model derived from 1D ^{1}H NMR spectral data of urine samples from male Hanover-Wistar rats treated with vehicle (control) or CCl_4 at 2.0 mL/kg.

ISSN 2249-3875

Table 5.8 OPLS-DA detected 1D ^{1}H NMR chemical shifts responsible for the separation of 1D ^{1}H NMR derived spectra in the urine of male Hanover-Wistar rats treated with vehicle (control) or CCl_4 at 2.0 mL/kg.

Chemical shift (δ), multiplicity	Endogenous metabolites	Change in urinary metabolite concentration (increase (+); decrease (-))
8.34-8.38 (s)	Formate	+*
7.74-7.78 (d)	Hippurate	-***
7.58-7.62 (t)	Hippurate	-**
7.54-7.58 (t)	Hippurate	-**
7.46-7.50	Unidentified	-***
7.26-7.30	Unidentified	-*
7.10-7.14	Unidentified	-**
6.54-6.58 (s)	Fumarate	-
4.42-4.46	Unidentified	+*
3.98-4.02	Unidentified	-
3.94-3.98	Unidentified	+*
3.86-3.90 (d)	Hippurate	-
3.78-3.82 (s)	Creatine	+*
3.74-3.78 (m)	Glucose	+
3.70-3.74	Unidentified	+
3.62-3.66 (m)	Glucose	+*
3.54-3.58 (m)	Glucose	+
3.50-3.54	Unidentified	+
3.34-3.38 (m)	Glucose	+*

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	3.30-3.34 (t)	Taurine	+*	
	3.22-3.26 (s)	TMAO	-	
	3.14-3.18 (t)	Taurine	+	
	3.02-3.06 (s)	Creatinine	-	
	2.94-2.98 (s)	Creatine	+	
	2.90-2.94 (t)	2-oxoglutarate	+	
	2.62-2.66 (d)	Citrate	+	
	2.58-3.62	Unidentified	+*	
	2.46-2.50	Unidentified	+*	
	2.42-2.46 (d)	Citrate	+	
	2.34-2.38 (t)	2-oxoglutarate	+	
	2.30-2.34 (s)	Succinate	+	
	2.22-2.26	Unidentified	-**	
	1.98-2.02	Unidentified	-**	
	1.54-1.58	Unidentified	-**	

S, singlet; d, doublet; t, triplet; m, multiplet. TMAO=Trimethylamine N-oxide.

appeared to be slightly subdued in comparison to the control animals. One animal treated with 3.6 mL/kg CCl_4 had a hunched posture. At autopsy, i.e., 24 hours post-dosing, livers from CCl_4-treated animals appeared paler in colour when compared to the control livers and the change in colour was more pronounced as the CCl_4 dose level increased.

Body weights

Table shows the change in body weight (for both control and CCl_4-treated animals) during the 18 hour period while rats were in the metabolism cages. The mean change in body weight for CCl_4-treated animals was compared to the change in body weight for controls. Although all animals lost weight over the 18 hour period the decrease in body weight for animals in the 1.2 mL/kg dose level group was the greatest (-19.83 g). Animals dosed at 2.8 mL/kg lost the least weight, -9.03 g (**P<0.01). However, there appears to be no clear CCl_4 dose-related effect on body weight of the animals.

Liver weights

Figure shows the mean relative liver weights at each CCl_4 dose level. The liver weights for

CCl_4-treated animals were significantly increased over controls (**P<0.01) at 0.8 mL/kg and above, and there was a dose-related increase. At the highest dose level (3.6 mL/kg),

Table 5.9 Body weight change for male Hanover-Wistar rats treated with increasing doses of CCl_4 during an 18 hour period in metabolism cages..

Dose level of CCl_4 (mL/kg)	Mean (SD) change in body weight (g)
0	-16.12 (2.09)
0.4	-19.25 (1.95)
0.8	-15.13 (2.89)
1.2	-19.83 (2.16)
1.6	-16.33 (1.84)
2.0	-17.15 (2.32)
2.4	-9.23** (3.01)
2.8	-9.03** (3.77)
3.2	-12.22 (4.52)
3.6	-16.17 (2.58)

relative liver weights were approximately 2-fold greater than control animals (***P<0.001).

Kidney weights

The mean relative kidney weights for rats treated with CCl_4 were similar to control at the lower dose levels (0.4, 0.8, 1.2, 1.6 and 2.0 mL/kg CCl_4) (Figure 3.1 B). However, at 2.4 mL/kg the relative kidney weights (4.23 g/kg BW) were significantly greater than controls (3.64 mg/kg BW) (*P<0.05), and at the highest dose level (3.6 mL/kg) there was a 1.23-fold increase over control weights (**P<0.01). However, there was no significant difference in kidney weights at 2.8 and 3.2 mL/kg CCl_4 when compared to controls.

Weights of other organs

At post-mortem, the spleen, thymus, heart, adrenals and testes were removed and the relative weights calculated. There were no CCl_4-treatment related effects on the relative weights of the spleen, thymus and heart in this study. The mean relative adrenal weight was significantly decreased at 2.0 mL/kg compared to control animals (0.18 and 0.24 g/kg BW respectively; *P<0.05) but similar to control at all other dose levels. The relative mean weight of the testes was decreased by 19 % in the 2.8

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mL/kg group (**P<0.01) and by 16 % in the 3.6 mL/kg group (*P<0.05) when compared to control animals, however, in general, there was no CCl_4 dose-related trend.

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