Available online at www.sciencedirect.com
SCIENCE DIRECT
Toxicology and Applied Pharmacology 212 (2006) 35 – 44
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Metabonomic evaluation of idiosyncrasy-like liver injury in rats cotreated with ranitidine and lipopolysaccharide

Jane F. Maddox[a], James P. Luyendyk[a], Gregory N. Cosma[b,1], Alan P. Breau[c,2], Roy H. Bible Jr.[c], George G. Harrigan[d,3], Royston Goodacre[e], Patricia E. Ganey[a], Glenn H. Cantor[b,4], Gary L. Cockerell[b], Robert A. Roth[a,*]

[a]*Department of Pharmacology and Toxicology, National Food Safety and Toxicology Center, Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA*
[b]*Investigative Toxicology, Pharmacia Corporation, Kalamazoo, MI 49001, USA*
[c]*Pharmacia Corporation, Skokie, IL 60077, USA*
[d]*HTS Metabolic Profiling, Pharmacia Corporation, Chesterfield, MO 63198, USA*
[e]*School of Chemistry, The University of Manchester, Manchester, England, UK*

Received 19 April 2005; revised 20 June 2005; accepted 21 June 2005
Available online 27 July 2005

Abstract

Idiosyncratic liver injury occurs in a small fraction of people on certain drug regimens. The cause of idiosyncratic hepatotoxicity is not known; however, it has been proposed that environmental factors such as concurrent inflammation initiated by bacterial lipopolysaccharide (LPS) increase an individual's susceptibility to drug toxicity. Ranitidine (RAN), a histamine-2 receptor antagonist, causes idiosyncratic liver injury in humans. In a previous report, idiosyncrasy-like liver toxicity was created in rats by cotreating them with LPS and RAN. In the present study, the ability of metabonomic techniques to distinguish animals cotreated with LPS and RAN from those treated with each agent individually was investigated. Rats were treated with LPS or its vehicle and with RAN or its vehicle, and urine was collected for nuclear magnetic resonance (NMR)- and mass spectroscopy-based metabonomic analyses. Blood and liver samples were also collected to compare metabonomic results with clinical chemistry and histopathology. NMR metabonomic analysis indicated changes in the pattern of metabolites consistent with liver damage that occurred only in the LPS/RAN cotreated group. Principal component analysis of urine spectra by either NMR or mass spectroscopy produced a clear separation of the rats treated with LPS/RAN from the other three groups. Clinical chemistry (serum alanine aminotransferase and aspartate aminotransferase activities) and histopathology corroborated these results. These findings support the potential use of a noninvasive metabonomic approach to identify drug candidates with potential to cause idiosyncratic liver toxicity with inflammagen coexposure.

Keywords: Metabonomic analysis; Lipopolysaccharide; Liver injury; Drug idiosyncrasy

Introduction

Inflammation induced by bacterial lipopolysaccharide (LPS) enhances the hepatotoxicity of several xenobiotic agents in rats. Hepatotoxicants that show a reduced threshold for liver toxicity when given to rats in combination with LPS include aflatoxin B_1 (Luyendyk et al., 2002), allyl alcohol (Sneed et al., 1997), and monocrotaline (Yee et al., 2002). In addition, there is evidence that some pharmaceut-

* Corresponding author.
E-mail addresses: maddox@msu.edu (J.F. Maddox), rothr@msu.edu (R.A. Roth).
[1] Current address: Bristol-Myers Squibb Company, New Brunswick, NJ, USA.
[2] Current address: Amgen Inc., Thousand Oaks, CA, USA.
[3] Current address: Pfizer, Inc., Chesterfield, MO, USA.
[4] Current address: Bristol-Myers Squibb Company, Princeton, NJ, USA.

0041-008X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2005.06.02

ical agents are rendered hepatotoxic by coexposure to LPS, including halothane (Lind et al., 1984), cocaine (Labib et al., 2002), and chlorpromazine (Buchweitz et al., 2002), and this phenomenon is hypothesized to be related to idiosyncratic drug reactions seen with these agents (Roth et al., 2003).

Ranitidine (RAN) is a histamine-2 (H2) receptor antagonist available both by prescription and over-the-counter for treatment of duodenal ulcers and esophageal reflux. A small percentage of people taking RAN have developed idiosyncratic liver injury (Fisher and Le Couteur, 2001). Hepatotoxicity associated with RAN is characterized by increases in serum markers of hepatocellular injury with more modest increases in indicators of cholestatic injury and, typical of idiosyncrasy, the time of onset of hepatotoxicity relative to initiation of therapy varies greatly (Halparin, 1984; Hiesse et al., 1985). In a recent publication from this laboratory, we observed liver toxicity in rats coexposed to LPS and RAN but not in rats treated with either RAN or LPS alone (Luyendyk et al., 2003).

Metabonomics has been defined as "the quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999). Biofluid metabonomics has the potential to provide biochemical information on the status of intact tissues and live animals (Nicholson et al., 2002). Xenobiotic agents alter normal biochemical processes in organisms; these changes are reflected in the plasma and ultimately result in perturbations in urine composition. Small amounts of urine can be collected during the course of a study, and high-resolution ^{1}H nuclear magnetic resonance (NMR) spectra and mass spectra can be used to detect xenobiotic-induced changes. Metabonomic analysis could offer numerous advantages over conventional histopathologic or enzymatic protocols (Nicholson et al., 1999; Nicholson et al., 2002). Operationally, it involves systematic spectral analysis of urine to associate organ toxicity with specific spectral patterns and to identify surrogate markers of toxicity. A large number of endogenous metabolites can be monitored simultaneously, and alterations in the quantity or quality of the overall signals can be evaluated. Pattern recognition analyses applied to these data can simplify the large number of variables, and visualize differences among groups, enabling data clustering and revealing treatment differences (Lindon et al., 2004). A metabonomic approach has been used to distinguish three hepatotoxicants given to rats: alpha-naphthyl isothiocyanate (ANIT), D-(+)-galactosamine, and butylated hydroxytoluene, allowing differentiation among them by the unique metabolic patterns they induced in the urine (Beckwith-Hall et al., 1998). Rats treated with 1 of 2 renal toxicants (2-bromoethylamine or 4-aminophenol), or 1 of 2 hepatotoxicants (ANIT or carbon tetrachloride) were also identified as distinct from one another and from vehicle-treated rats by urine NMR metabonomics (Robertson et al., 2000).

We investigated the ability of metabonomic techniques to discern differences among rats treated with vehicle; an inflammation-inducing, but non-hepatotoxic dose of LPS; a non-hepatotoxic dose of RAN; and cotreatment with these doses of LPS and RAN. Only the last treatment produces liver injury when given together. We used NMR analysis and compared results with mass spectroscopic analysis, which represents a complementary approach to NMR, potentially offering advantages in throughput and reduced sample requirements.

Methods

Animals

Male, Sprague–Dawley rats [Crl:CD (SD)IGS BR; Charles River, Portage, MI] weighing 250–300 g were housed individually in plastic metabolism cages for these studies. Rats received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (1996), and approval was obtained from the Michigan State University Animal Care and Use Committee. They were fed powdered certified rat diet (Labdiet 5002, Purina Mills, Inc., St. Louis, MO), allowed access to water ad libitum and were acclimated for 5 days in standard group rat laboratory cages and for 2 days in metabolism cages prior to treatment. From group housing (3 rats per cage), animals were randomly assigned to individual metabolism cages and treatment groups. The light/dark cycle was 12 h, with lights on from 6 am to 6 pm.

Experimental protocol

Rats fasted for 24 h were given 44.4×10^{6} EU/kg LPS (*Escherichia coli* serotype O55:B5, Sigma-Aldrich Corp., St. Louis, MO) or its saline vehicle, intravenously at 8 am. 2 h later, 30 mg/kg RAN or sterile phosphate-buffered saline (PBS) vehicle was administered intravenously. RAN solution was administered at 2 mL/kg at a rate of approximately 0.15 mL/min.

At selected time intervals, urine samples from each rat were collected over ice with the addition of sodium azide (0.1% w/v urine). Samples were collected from: −22 to −16 h (10 am to 4 pm), −16 to −10 h (4 pm to 10 pm), and −10 to 0 h (10 pm to 8 am) during fasting (pretreatment) and 2 to 8 h (10 am to 4 pm), 8 to 14 h (4 pm to 10 pm), and 14 to 26 h (10 pm to 10 am) relative to LPS treatment. Urine samples were centrifuged at 2000 rpm for 10 min to remove particulate matter, and the samples were stored at −80 °C until analysis. Twenty-four hours after RAN treatment, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and killed by exsanguination. Blood was allowed to clot at room

temperature; serum was collected and stored at −20 °C until use. Representative (3–4 mm) slices of the ventral half of the left lateral liver lobe were collected and fixed in 10% neutral buffered formalin.

Clinical chemistry

Selected serum enzymes were analyzed using a Beckman-Coulter (Brea, CA) Synchron CX7 random access clinical analyzer powered by version 6.4 CX7 software. Hepatic parenchymal cell injury was estimated as increases in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities.

^{1}H NMR spectroscopy of urine

A buffer solution (pH 7.5) was prepared by dissolving 5.77 g of Na_2HPO_4 (anhydrous) and 1.05 g NaH_2PO_4 (anhydrous) in a mixture of 140 mL of water and 60 mL of D_2O. To this solution was added 34.5 mg of 3-trimethylsilyl-1-[2,2,3,3,-$^2H_4$]propionate to provide a reference standard for NMR resonance, set at 0.0 ppm, and 38 mg of sodium azide to inhibit bacteria that could degrade the sample.

A 0.2-mL aliquot of the buffer solution was added to a 0.4-mL sample of the urine from each collection period. After removal of precipitate, the proton NMR spectra were determined using a Bruker DRX-600 spectrometer (Bruker Instruments, Billerica, MA, USA) with water suppression using a 1-D Nuclear Overhauser Enhancement Spectroscopy (NOESY) presaturation pulse sequence. The phase was corrected, and the baseline was flattened.

Data analysis. Using the Bruker computer program AMIX, each NMR spectrum was reduced to 245 integrated regions of equal width (0.04 ppm). The region containing the water suppression and the cross relaxation caused by urea (4.50–5.98 ppm) was removed. Using SIMCA-P version 8.0 software (Umetrics, Umeå, Sweden), the spectra were then normalized to a constant integrated intensity of 100 units to compensate for variations in urine concentration and facilitate comparison among animals. Additionally, peaks related to RAN were removed (2.82, 2.86, 2.90, 3.46, 3.86, 4.26, 6.38, 6.66, and 6.86 ppm). The data were then unit-scaled, and the mean of each variable was subtracted from each column of the data set. Principal component analysis (PCA) was then applied to the entire data set of the various treatment groups. The principal components (PCs) that account for the most variance are shown.

Direct infusion electrospray mass spectrometry (DIEMS)

DIEMS was performed on urine using an Agilent 1100 Series Liquid Chromatograph/Mass Selective Detector system (Agilent Technologies, Palo Alto, CA, USA). Samples (1 μL injection) were prepared (20- to 40-fold dilution) in 60% aqueous acetonitrile containing 0.01% trifluoroacetic acid (TFA) and infused into the mass spectrometer at a flow rate of 75 μl min^{-1} using an Agilent 1100 Series capillary high-performance liquid chromatograph (HPLC) system operating in flow injection mode. The mobile phase was 60% aqueous acetonitrile containing 0.01% TFA. Spectra were collected in the positive ion mode with fragmentor voltage set at 50 V and stepsize set to 0.4 atomic mass units (amu). Capillary voltage was set at 4000 V. The desolvation gas flow rate was kept at 420 l h^{-1} and the drying gas temperature at 250 °C. The mass range was set to m/z 125–450, and spectra were typically collected for 1 min; these were then summed. Normalization was achieved by plotting peak abundance as a percentage of the total area, and resulting data were used for subsequent cluster analysis and construction of a dendrogram.

Principal components analysis. PCA was performed on the DIEMS second derivative data set to give a new set of orthogonal variables called Principal Components (PCs), the first few of which typically account for >95% of the variance (Causton, 1987; Jolliffe, 1986).

Discriminant function analysis (DFA). DFA is a supervised projection method (Manly, 1994). A priori information about sample grouping in the data set is used to produce measures of within-group variance and between-group variance. This information is then used to define discriminant functions that optimally separate the groups a priori (herein the groups were defined as instrument replicates). In this study, the first 10–20 PC scores are used as the data source for DFA.

Statistical analyses

Results are presented as mean ± SEM. For serum clinical chemistry data, two-way analysis of variance (ANOVA) was employed after appropriate data transformation. All individual group comparisons were made using Tukey's method. The criterion for significance was $P < 0.05$ for all studies.

Results

Clinical chemistry

LPS or RAN treatment alone did not cause significant liver injury, as evidenced by no change in serum ALT (Fig. 1A) or AST (Fig. 1B) activities in either treatment group. However, treatment with LPS followed 2 h later with RAN caused significant parenchymal cell injury within 24 h, with concomitant increases in ALT and AST activities.

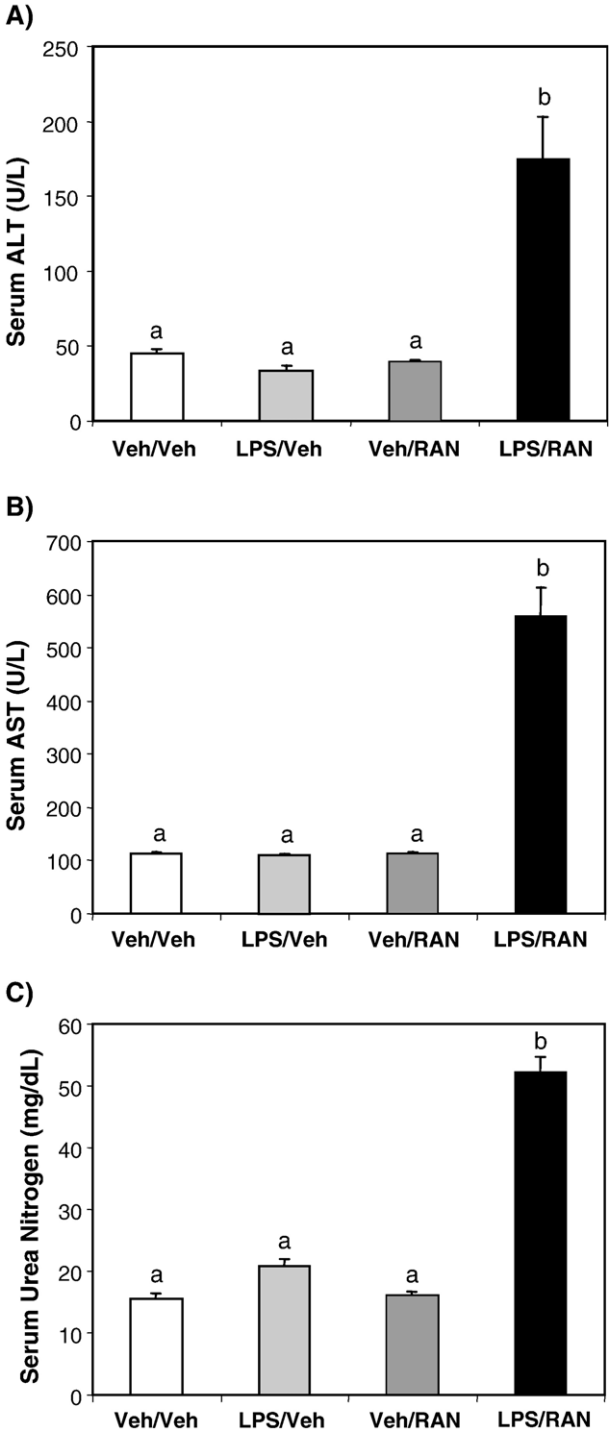

Fig. 1. Hepatotoxicity from LPS/RAN cotreatment. Rats were treated with 44.4×10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). Hepatic parenchymal cell injury was estimated 24 h after RAN administration by increases in serum (A) ALT and (B) AST activities. (C) Renal injury and hydration status were estimated from increases in serum BUN activity. $n = 5–7$ rats per group. Data are expressed as mean ± SEM. Bars with different letters are significantly different from one another ($P < 0.05$).

Statistical analyses revealed a significant interaction between LPS and RAN treatments in the increase of both ALT and AST activities. Serum urea nitrogen was also increased in rats in the LPS/RAN treatment group (Fig. 1C) and was likely caused by prerenal azotemia due to dehydration, since another serum marker of renal dysfunction (i.e., creatinine) was not increased substantially (data not shown).

^{1}H NMR spectroscopy of urine samples

Rats in the LPS/RAN cotreated group did not urinate during the first collection period after treatment (0 to 8 h); therefore, all metabonomic comparisons among treatment groups were made among the urine collection periods from 8 to 14 h and 14 to 26 h after LPS treatment. Urine from rats treated with LPS/RAN showed consistent changes in ^{1}H NMR spectra in both the 8- to 14-h (data not shown) and 14- to 26-h collection periods (Fig. 2) compared with pretreatment samples. The predominant changes included decreases in citrate, hippurate, and 2-oxoglutarate, and increases in acetate, creatine, creatinine, taurine, and trimethylamine *N*-oxide (Table 1). These changes are consistent with those seen by other investigators in animals with experimental hepatocellular injury (Beckwith-Hall et al., 2002; Clayton et al., 2003). ^{1}H NMR spectra of urine from vehicle- and RAN-treated rats showed only modest changes in endogenous metabolite levels over the 48-h collection period. Urine metabolite spectra from rats treated with LPS alone varied little compared to pretreatment controls up to 14 h posttreatment. However, during the 14- to 26-h posttreatment collection period, creatine increased slightly and citrate and 2-oxoglutarate decreased moderately in LPS-treated rats compared with their pretreatment controls (data not shown).

PCA of metabolite spectra

PCA was performed on urine NMR spectra from all animals within a collection period. Score plots of the urine collected from 8 to 14 h posttreatment illustrating PC1 versus PC2 (Fig. 3) show that the LPS/RAN-treated animals clearly separated from vehicle-treated, RAN-treated, and LPS-treated groups. For the urine collection period from 14 to 26 h posttreatment, the NMR PCA revealed separation of some animals in the RAN/LPS-treated group; however, the LPS-alone group showed some overlap with the cotreated group on the PC1 versus PC2 plot (Fig. 4). The vehicle- and RAN-treated groups clearly segregated from the LPS-treated groups.

Metabolic trajectories of ^{1}H NMR data

Urine samples collected from 8 through 14 h and from 14 through 26 h were used to plot metabolic trajectories, a method of visualizing changes in metabolic profiles with time. The trajectories were distinctly different among treatment groups (Fig. 5). The individual animals treated

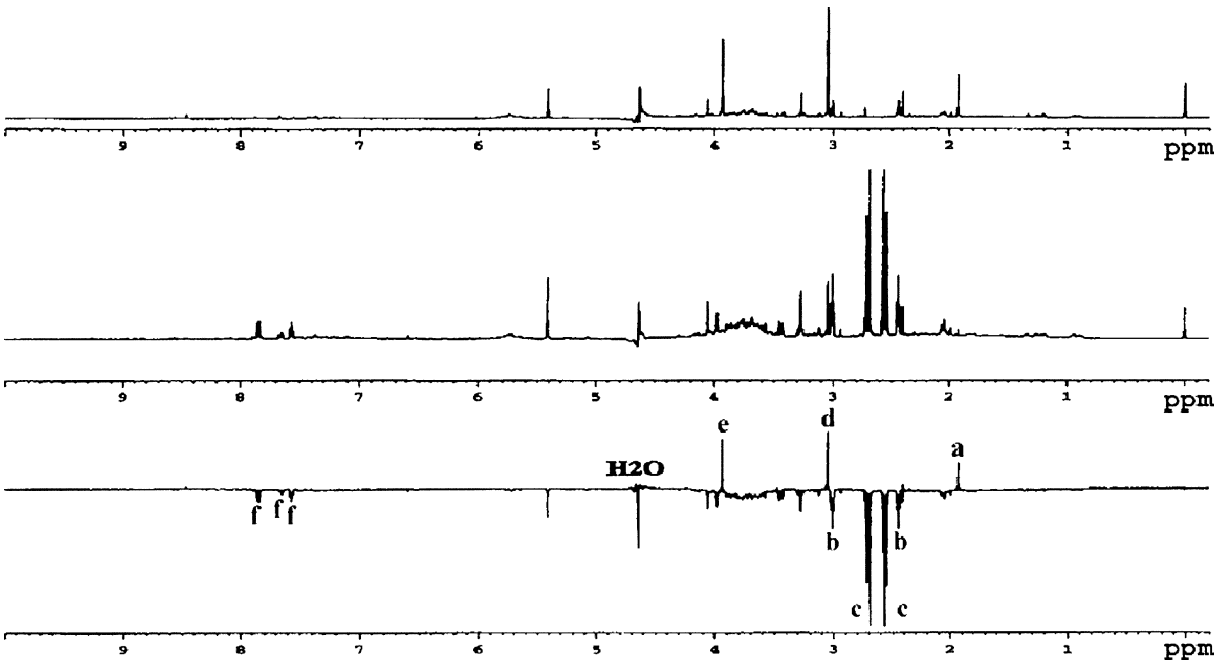

Fig. 2. NMR spectra of urine. Rats were treated with 44.4 × 10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). ^{1}H NMR spectra of urine from one LPS/RAN-treated rat: 14 through 26 h after LPS treatment (top), 16 through 22 h before LPS treatment (middle), and posttreatment spectrum minus pretreatment spectrum (bottom). Peaks showing major differences between pre- and posttreatment are lettered and the components identified: (a) acetate; (b) 2-oxoglutarate; (c) citrate; (d) creatinine; (e) creatine; (f) hippurate.

with LPS/RAN exhibited trajectories that moved in the same direction, suggesting a common set of biochemical changes in the individuals in that group. Moreover, the changes were larger in magnitude than in the trajectories from other groups. Trajectories for LPS/Veh-treated rats also separated from the other groups, but the changes were smaller in magnitude than those in the LPS/RAN group and traversed different directions from one another within the group. Both Veh/RAN and Veh/Veh trajectories grouped together and, like the LPS/Veh group, showed less change between the sample collection times and ran in dissimilar directions.

Table 1
Changes in endogenous urinary metabolites 14 to 26 h after LPS/RAN treatment

Endogenous metabolite	Increase (↑) or decrease (↓)
Hippurate	↓
Citrate	↓
2-Oxoglutarate	↓
Trimethylamine oxide	↑
Creatine	↑
Creatinine	↑
Taurine	↑
Acetate	↑

Rats were treated with 44.4 × 10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). Urine was collected pretreatment (−22 to −16 h) and posttreatment (14 to 26 h) and ^{1}H NMR spectra were obtained as described in Methods. Pretreatment spectra were subtracted from posttreatment spectra, and the change is represented in the table.

DIEMS analysis of urine samples

The discriminatory potential of DIEMS to evaluate closely related populations was assessed by comparing urine collected at three different times during the predose period. The predose samples offered a larger data set (n = 23) than that for the drug- and LPS-treated subsets (n = 5), and all rats at this time (predosing) were handled similarly and were unexposed to xenobiotic agents. Differences might arise over time due to changes in nutritional status (food was removed at the beginning of the predose period). Discriminant function analysis separated samples from untreated, predose groups by time of sampling (data not shown).

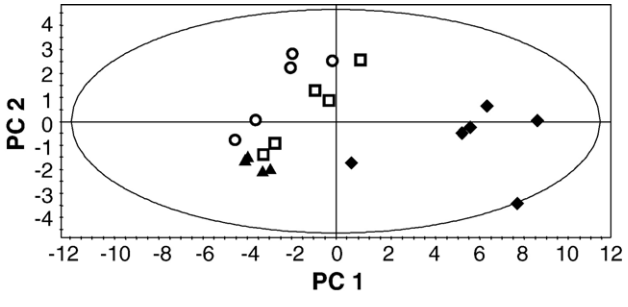

Fig. 3. NMR PCA score plot of 8- to 14-h posttreatment urine samples. Rats were treated with 44.4 × 10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). Urine was collected 8 through 14 h after administration of LPS or its vehicle. PCA was performed on all urine samples collected. The first two principal components (PC1 and PC2) are plotted. Veh/Veh (▲); Veh/RAN (○); LPS/Veh (□); LPS/RAN (♦).

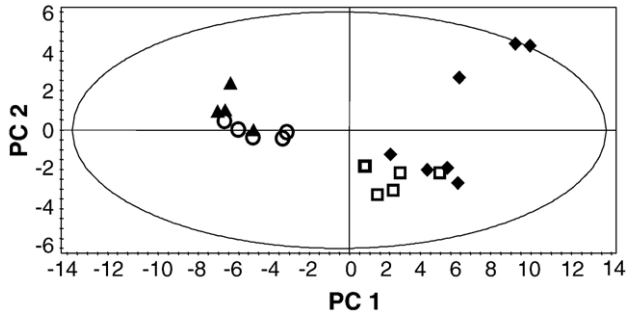

Fig. 4. NMR PCA score plots of 14- to 26-h posttreatment urine samples. Rats were treated with 44.4 × 10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). Urine was collected 14 through 26 h after administration of LPS or its vehicle. PCA was performed on all urine samples collected. The first two principal components (PC1 and PC2) are plotted. Veh/Veh (▲); Veh/RAN (○); LPS/Veh (□); LPS/RAN (♦).

Despite this initial demonstration that three closely related populations differing only in the time of day in which they were collected could be discriminated by MS analysis, MS must discriminate toxic from nontoxic treatments to be of value. A discriminant function analysis of the untreated controls and the 14- to 26-h posttreatment groups revealed clear separation of all of the groups (Fig. 6). To test further the analysis model, one of the biological replicates from each of the four treatment groups was projected into the resultant PC-DFA model created from the rest of the data. This validated discriminant analysis of the data clustered each treatment group more tightly and distinguished RAN, LPS, and vehicle-alone treatments from one another (Fig. 7). The test spectra (those that were projected, marked with an asterisk) were recovered with their correct clusters. However, it is also evident from this figure that

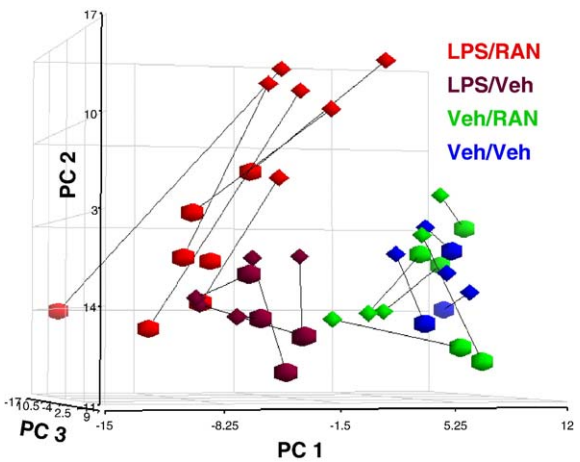

Fig. 5. PC map of NMR-defined metabolic trajectories. Rats were treated with 44.4 × 10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). Urine was collected 8 through 14 h and 14 through 26 h after administration of LPS or its vehicle. PCA was performed on all urine samples. Data for individual animals are plotted as the first three principal components (PC1, PC2, and PC3). Lines connect the values for the 8- to 14-h collection period and 14- to 26-h collection period for each animal. The length of the line approximates the degree of difference over time. 8–14 h (♦); 14–26 h (●).

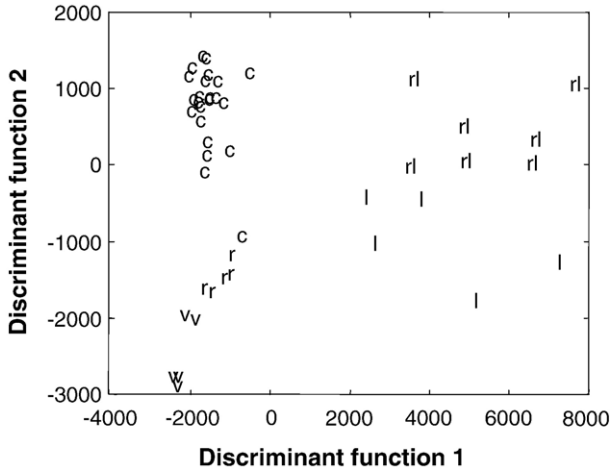

Fig. 6. Discriminant function analysis (DFA) of urine analyzed by DIEMS. Rats were treated with 44.4 × 10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). Urine was collected −10 to 0 h before treatment (predose controls) or 14 through 26 h after administration of LPS or its vehicle. The first 10 principal components (PCs) were used to construct this PC-DFA plot, and these explained 94.9% of the total variance. The a priori group structure used was the instrument replicate spectra (i.e., there were 45 samples). The means of the replicate spectra are shown. Untreated predose controls (c) (n = 23), Veh/Veh-treated (v) (n = 5), Veh/RAN-treated (r) (n = 5), LPS/Veh-treated (l) (n = 5), and LPS/RAN-treated (rl) (n = 7) populations.

there is greater heterogeneity in the LPS-treated populations. It is still possible to discriminate the hepatotoxic cotreatment (LPS/RAN) from LPS alone, which was not hepatotoxic, based on changes in ALT activity (Fig. 1). In addition, a dendrogram was constructed as described above and

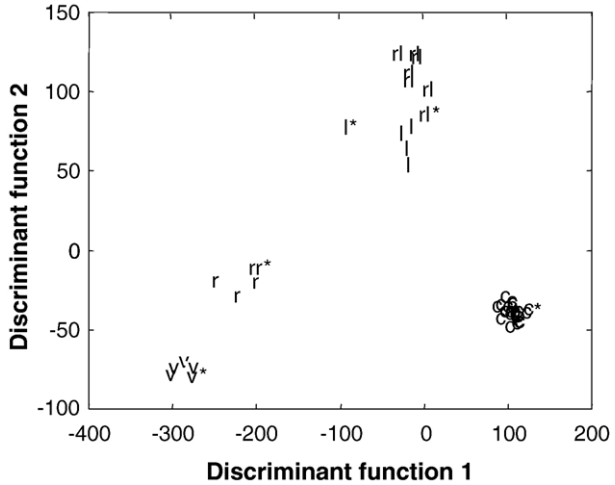

Fig. 7. Validated discriminant function analysis (DFA) of urine analyzed by DIEMS. Rats were treated with 44.4 × 10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). Urine was collected −10 to 0 h before treatment (predose controls) or 14 through 26 h after administration of LPS or its vehicle. Untreated predose controls (c) (n = 23), Veh/Veh-treated (v) (n = 5), Veh/RAN-treated (r) (n = 5), LPS/Veh-treated (l) (n = 5), and LPS/RAN-treated (rl) (n = 7) populations. The samples marked with an asterisk were projected into PC-DFA space constructed as detailed in the text.

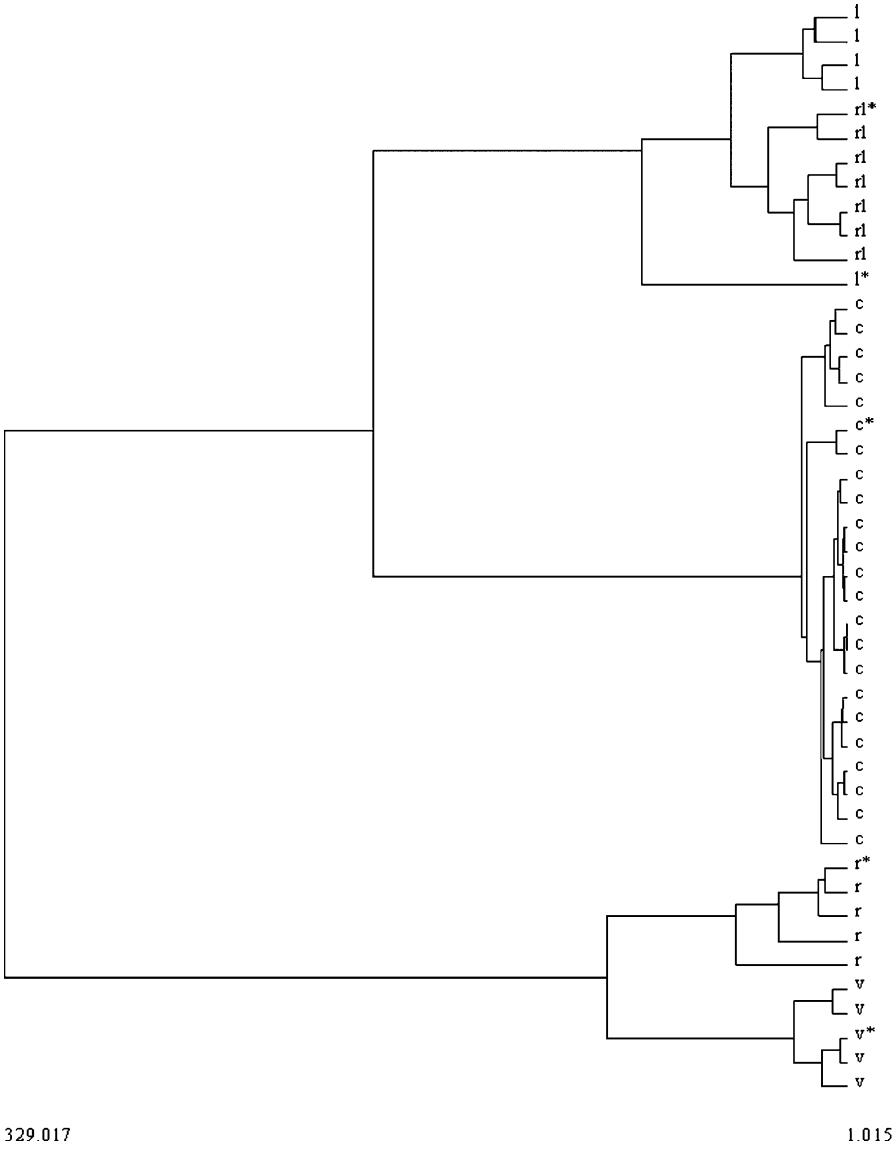

Fig. 8. Validated hierarchical cluster analysis (HCA) of urine analyzed by DIEMS. Rats were treated with 44.4×10^6 EU/kg LPS or its vehicle (iv), then 2 h later with 30 mg/kg RAN or its vehicle (iv). Urine was collected −10 to 0 h before treatment (predose controls) or 14 through 26 h after administration of LPS or its vehicle. Untreated predose controls (c) (n = 23), Veh/Veh-treated (v) (n = 5), Veh/RAN-treated (r) (n = 5), LPS/Veh-treated (l) (n = 5), and LPS/RAN-treated (rl) (n = 7) populations. The samples marked with an asterisk were projected into PC-DFA space prior to HCA.

segregation of the treatment groups is more apparent in the dendrogram (Fig. 8).

Discussion

A previous study indicated that idiosyncrasy-like injury develops in rats when they are cotreated with LPS and RAN (Luyendyk et al., 2003). This is documented by changes in serum levels of liver enzymes and histopathologic changes in the liver seen only in the cotreated animals (Luyendyk et al., 2003). In confirmation, similar changes in liver enzymes were observed in these experiments (Fig. 1) and histopathologic changes in livers were comparable to previous studies (data not shown). In the current investigation, our hypothesis was that metabonomic evaluation of urine could distinguish rats cotreated with LPS and RAN under conditions that lead to hepatotoxicity from those treated with vehicle or either agent singly.

Previously, metabonomic analysis using NMR has been shown to separate animals treated with single nephro- or hepato-toxicants based on urine metabolites (Robertson et al., 2000; Beckwith-Hall et al., 1998), and to have some ability to differentiate doses of hepatotoxicants (Robertson et al., 2000). Metabonomics has also been proposed as a tool for screening of drugs for toxicity in drug discovery programs (Holmes et al., 2001). This is the first time, to our knowledge, that metabonomics has been used to discriminate between animals treated with a single compound from those cotreated with a second.

The urine from those rats cotreated with LPS/RAN showed metabolic changes that were not seen in urine from the rats treated with either vehicle, LPS, or RAN alone. Urinary metabolite changes in LPS/RAN-treated rats experiencing hepatotoxicity included increases in taurine and creatine and decreases in citrate and 2-oxoglutarate (Fig. 2 and Table 1). These changes have been associated with other xenobiotic agents that are liver toxicants (Beckwith-Hall et al., 1998). Animals treated with a non-hepatotoxic dose of RAN alone did not segregate from vehicle-treated rats at either the 8- to 14-h or 14- to 26-h times posttreatment. In the 8- to 14-h posttreatment period, the rats given the non-hepatotoxic LPS dose separated from vehicle- and RAN-treated samples, as well as from the LPS/RAN cotreated samples on PC plots. In these singly treated groups, as well as vehicle control, there was no increase in urinary taurine or creatine, but citrate and 2-oxoglutarate decreased. Increases in taurine and creatine are thought to be more specific to hepatoxicity than decreases in citrate and 2-oxoglutarate, which may simply indicate changes in energy metabolism (Beckwith-Hall et al., 1998). There was greater discrimination between animals treated with or without LPS than between the LPS-alone and LPS/RAN groups in the 14- to 26-h time period when evaluated using PCA or DFA (Figs. 4, 6, and 7). The individual LPS/RAN-treated rats with the greatest serum ALT activities at 26 h were not those with the largest metabonomic changes (data not shown). This may be because the urine analysis reflects the average changes in metabolism over a 12-h period, whereas ALT values represent a snapshot in time. For example, if liver injury in some rats peaked early during the 14- to 26-h period, and injury had begun to resolve, ALT values at the time of collection of blood at 26 h may be smaller than they were earlier. Nonetheless, the metabolic changes associated with peak liver injury would be captured in the urine produced early in the collection period. Some rats may have been at different stages in the development and resolution of liver damage from LPS/RAN, but further investigation will be required to address this possibility. Alternatively, differences between the magnitude of metabonomic responses and ALT activity in serum could arise because metabonomics analyzes many alterations in organism biochemistry, whereas ALT more specifically detects injured plasma membranes, particularly for hepatocytes.

The metabolic trajectories with time of the urine also produced results that support our hypothesis. Urine from LPS/RAN-treated rats showed similar geometric trajectories, both in size and direction. This suggests that the biochemical responses and endogenous metabolites excreted by those rats were similar in character (Keun et al., 2004). In contrast, the groups treated with LPS, RAN, or vehicle alone demonstrated dissimilar geometry in their trajectories, though each group generally occupied a similar multi-dimensional space. Both the direction and magnitude of change between the points varied greatly within each group, indicating that the profile of urine metabolites differed among animals at these times.

Other spectral approaches to the metabolic profiling of biofluids are gaining use, including the use of LC–MS (Plumb et al., 2002). However, the pharmaceutical industry continues to drive toward methods to analyze complex biological systems rapidly and this necessitates high-throughput approaches. Current approaches to LC–MS are compromised by long run-times (typically >10 min). We were therefore intrigued by the possibility of using DIEMS as a prioritization tool or filter for more detailed LC–MS-based analyses: in other words, expanding its use as a high-throughput "work-horse" of MS-based metabonomics. DIEMS has been used in microbial characterization and, most recently, for high-throughput phenotypic classification of yeast mutants to support a functional genomics program (Allen et al., 2003; Vaidyanathan et al., 2001, 2002a, 2002b). The purpose of DIEMS is to generate a spectral fingerprint that is a function of the composition of the analyte mixture, but it is not specifically intended for metabolite identification or quantification. Its ability to discriminate at the subspecies level when applied to microbial systems suggests that it could be of value in discriminating fluids such as urine that may differ markedly due to toxicological insult, but with the understanding that chromatographic separations would be necessary in follow-up identification strategies.

Reports on applications of LC/MS to toxicology demonstrate the potential of LC/MS for screening rat urine, including at least one study showing that it is possible to differentiate spectra of urine samples from control rats and those of rats treated with xenobiotic agents (Plumb et al., 2002). Moreover, components (m/z values) that contribute to separation can be identified and chemical structures assigned. The small sample volumes would also allow, at least in principle, studies on biofluids such as cerebrospinal fluid (e.g., for neuropathological analysis) that are present in only small amounts in typical animal models used in toxicology studies. Additionally, signals from components such as drug vehicle, residual drug, and drug-related metabolites are often present in NMR spectra of urine from treated animals and must be electronically filtered (Lindon et al., 2003). In principle, such 'lost" NMR data can be replaced with archive control data (Ebbels et al., 2004); however, potential biomarker signals present in this region are deleted. With MS, there is more distinct separation of peaks, such that drug-related peaks could be more readily removed, provided they are correctly identified. These qualities of MS-based analysis may make it an attractive additional technique, particularly for small volume samples or those with large concentrations of drug-related components. Despite its attractiveness, issues still remain with this approach. Firstly, the use of chromatography introduces a source of potential error due to retention time drift. Secondly, as mentioned above, reported run-times for LC/

MS are typically 10 min, and for LC/MS/MS 30 min is typical. This is appreciably longer than the run-time for NMR spectral acquisitions (1–5 min) and Fourier-transform infrared spectroscopy (FT-IR). Peak definition in urine analysis is often poor, and broad overlapping peaks are not unusual. Polar compounds, often cited as biomarkers of toxicity, can be difficult to analyze by reverse-phase LC–MS as they elute at the solvent front. As such, alternative HPLC methodologies such as ion-exchange could be incorporated, but maximizing coverage of urinary metabolites might necessitate several analytical LC platforms, with a concomitant decrease in throughput. Recent developments in Ultra-Pressure LC (UPLC) might serve to increase the throughput of MS analyses without requiring DIEMS (Plumb et al., 2004; Wilson et al., 2005). It is inevitable that developments in separation sciences and measurement technologies will drive the application of MS, either in direct infusion mode or coupled with novel chromatographic steps, in the profiling of complex biological matrices.

In our experiments, DIEMS was able to separate treatment groups using discriminant function analysis (Figs. 6 and 7). In addition, a dendrogram constructed from hierarchical cluster analysis grouped rats from the same treatments as most closely related, with the exception of one outlier animal from the LPS/Veh group (Fig. 8). Indeed, the cluster patterns appear superficially similar to those obtained from NMR analyses, in principle validating the DIEMS.

Overall, the results from these studies demonstrate that both NMR- and MS-based metabonomic evaluation of urine can distinguish an idiosyncrasy-like hepatotoxic reaction to cotreatment with an inflammagen (LPS) and a drug (RAN) from responses to either drug or inflammation alone. These data reinforce a previous study that demonstrated the ability of a novel technique for metabonomic evaluation of urine, i.e., FT-IR, to discriminate LPS/RAN cotreated rats from the other treatment groups (Harrigan et al., 2004). Even those rats for which LPS/RAN cotreatment caused no increase in serum ALT activity above the normal range were segregated by PCA using NMR- and MS-based urine analyses. This suggests that metabonomic evaluation might be a more sensitive marker of the potential for idiosyncratic drug toxicity than increases in serum liver enzyme activity. Further investigation with comparisons of metabonomics with serum chemistry and histopathology in this paradigm will be necessary to support this possibility fully.

Acknowledgments

This work was supported by grants from the Pharmacia Corporation and the National Institutes of Health (DK061315). The authors thank Chi Yan J. Tam for her technical support in NMR spectroscopy. RG thanks the UK Biotechnology and Biological Sciences Research Council for financial support.

References

Allen, J., Davey, H.M., Broadhurst, D., Heald, J.K., Rowland, J.J., Oliver, S.G., Kell, D.B., 2003. High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. Nat. Biotechnol. 21, 692–696.

Beckwith-Hall, B.M., Nicholson, J.K., Nicholls, A.W., Foxall, P.J., Lindon, J.C., Connor, S.C., Abdi, M., Connelly, J., Holmes, E., 1998. Nuclear magnetic resonance spectroscopic and principal components analysis investigations into biochemical effects of three model hepatotoxins. Chem. Res. Toxicol. 11, 260–272.

Beckwith-Hall, B.M., Holmes, E., Lindon, J.C., Gounarides, J., Vickers, A., Shapiro, M., Nicholson, J.K., 2002. NMR-based metabonomic studies on the biochemical effects of commonly used drug carrier vehicles in the rat. Chem. Res. Toxicol. 15, 1136–1141.

Buchweitz, J.P., Ganey, P.E., Bursian, S.J., Roth, R.A., 2002. Underlying endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy? J. Pharmacol. Exp. Ther. 300, 460–467.

Causton, D.R., 1987. A Biologist's Advanced Mathematics. Allen and Unwin, London.

Clayton, T.A., Lindon, J.C., Everett, J.R., Charuel, C., Hanton, G., Le, N.J., Provost, J.P., Nicholson, J.K., 2003. An hypothesis for a mechanism underlying hepatotoxin-induced hypercreatinuria. Arch. Toxicol. 77, 208–217.

Ebbels, T.M., Holmes, E., Lindon, J.C., Nicholson, J.K., 2004. Methods for spectral analysis and their applications: spectral replacement (6,683,455). Ref Type: Patent.

Fisher, A.A., Le Couteur, D.G., 2001. Nephrotoxicity and hepatotoxicity of histamine H2 receptor antagonists. Drug Saf. 24, 39–57.

Halparin, L.S., 1984. Adverse effects of ranitidine therapy. Can. Med. Assoc. J. 130, 668–672.

Harrigan, G.G., LaPlante, R.H., Cosma, G.N., Cockerell, G., Goodacre, R., Maddox, J.F., Luyendyk, J.P., Ganey, P.E., Roth, R.A., 2004. Application of high-throughput Fourier-transform infrared spectroscopy in toxicology studies: contribution to a study on the development of an animal model for idiosyncratic toxicity. Toxicol. Lett. 146, 197–205.

Hiesse, C., Cantarovich, M., Santelli, C., Francais, P., Charpentier, B., Fries, D., Buffet, C., 1985. Ranitidine hepatotoxicity in renal transplant patient. Lancet 1, 1280.

Holmes, E., Nicholson, J.K., Tranter, G., 2001. Metabonomic characterization of genetic variations in toxicological and metabolic responses using probabilistic neural networks. Chem. Res. Toxicol. 14, 182–191.

Jolliffe, I.T., 1986. Principal Component Analysis. Springer-Verlag, New York.

Keun, H.C., Ebbels, T.M., Bollard, M.E., Beckonert, O., Antti, H., Holmes, E., Lindon, J.C., Nicholson, J.K., 2004. Geometric trajectory analysis of metabolic responses to toxicity can define treatment specific profiles. Chem. Res. Toxicol. 17, 579–587.

Labib, R., Turkall, R., Abdel-Rahman, M.S., 2002. Endotoxin potentiates the hepatotoxicity of cocaine in male mice. J. Toxicol. Environ. Health, Part A 65, 977–993.

Lind, R.C., Gandolfi, A.J., Sipes, I.G., Brown, B.R.J., 1984. The involvement of endotoxin in halothane-associated liver injury. Anesthesiology 61, 544–550.

Lindon, J.C., Nicholson, J.K., Holmes, E., Antti, H., Bollard, M.E., Keun, H., Beckonert, O., Ebbels, T.M., Reily, M.D., Robertson, D., Stevens, G.J., Luke, P., Breau, A.P., Cantor, G.H., Bible, R.H., Niederhauser, U., Senn, H., Schlotterbeck, G., Sidelmann, U.G., Laursen, S.M., Tymiak, A., Car, B.D., Lehman-McKeeman, L., Colet, J.M., Loukaci, A., Thomas, C., 2003. Contemporary issues in toxicology the role of metabonomics in toxicology and its evaluation by the COMET project. Toxicol. Appl. Pharmacol. 187, 137–146.

Lindon, J.C., Holmes, E., Bollard, M.E., Stanley, E.G., Nicholson, J.K., 2004. Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. Biomarkers 9, 1–31.

Luyendyk, J.P., Shores, K.C., Ganey, P.E., Roth, R.A., 2002. Bacterial lipopolysaccharide exposure alters aflatoxin B1 hepatotoxicity: benchmark dose analysis for markers of liver injury. Toxicol. Sci. 68, 220–225.

Luyendyk, J.P., Maddox, J.F., Cosma, G.N., Ganey, P.E., Cockerell, G.L., Roth, R.A., 2003. Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. J. Pharmacol. Exp. Ther. 307, 9–16.

Manly, B.F.J., 1994. Multivariate Statistical Methods: A Primer. Chapman and Hall, London.

Nicholson, J.K., Lindon, J.C., Holmes, E., 1999. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29, 1181–1189.

Nicholson, J.K., Connelly, J., Lindon, J.C., Holmes, E., 2002. Metabonomics: a platform for studying drug toxicity and gene function. Nat. Rev., Drug Discov. 1, 153–161.

Plumb, R.S., Stumpf, C.L., Gorenstein, M.V., Castro-Perez, J.M., Dear, G.J., Anthony, M., Sweatman, B.C., Connor, S.C., Haselden, J.N., 2002. Metabonomics: the use of electrospray mass spectrometry coupled to reversed-phase liquid chromatography shows potential for the screening of rat urine in drug development. Rapid Commun. Mass Spectrom. 16, 1991–1996.

Plumb, R., Castro-Perez, J., Granger, J., Beattie, I., Joncour, K., Wright, A., 2004. Ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 18, 2331–2337.

Robertson, D.G., Reily, M.D., Sigler, R.E., Wells, D.F., Paterson, D.A., Braden, T.K., 2000. Metabonomics: evaluation of nuclear magnetic resonance (NMR) and pattern recognition technology for rapid in vivo screening of liver and kidney toxicants. Toxicol. Sci. 57, 326–337.

Roth, R.A., Luyendyk, J.P., Maddox, J.F., Ganey, P.E., 2003. Inflammation and drug idiosyncrasy—Is there a connection? J. Pharmacol. Exp. Ther. 307, 1–8.

Sneed, R.A., Grimes, S.D., Schultze, A.E., Brown, A.P., Ganey, P.E., 1997. Bacterial endotoxin enhances the hepatotoxicty of allyl alcohol. Toxicol. Appl. Pharmacol. 144, 77–87.

Vaidyanathan, S., Rowland, J.J., Kell, D.B., Goodacre, R., 2001. Discrimination of aerobic endospore-forming bacteria via electrospray-ionization mass spectrometry of whole cell suspensions. Anal. Chem. 73, 4134–4144.

Vaidyanathan, S., Kell, D.B., Goodacre, R., 2002a. Flow-injection electrospray ionization mass spectrometry of crude cell extracts for high-throughput bacterial identification. J. Am. Soc. Mass Spectrom. 13, 118–128.

Vaidyanathan, S., Winder, C.L., Wade, S.C., Kell, D.B., Goodacre, R., 2002b. Sample preparation in matrix-assisted laser desorption/ionization mass spectrometry of whole bacterial cells and the detection of high mass (>20 kDa) proteins. Rapid. Commun. Mass Spectrom. 16, 1276–1286.

Wilson, I.D., Plumb, R., Granger, J., Major, H., Williams, R., Lenz, E.M., 2005. HPLC-MS-based methods for the study of metabonomics. J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 817, 67–76.

Yee, S.B., Kinser, S., Hill, D.A., Barton, C.C., Hotchkiss, J.A., Harkema, J.R., Ganey, P.E., Roth, R.A., 2002. Synergistic hepatotoxicity from coexposure to bacterial endotoxin and the pyrrolizidine alkaloid monocrotaline. Toxicol. Appl. Pharmacol. 166, 173–185.