



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

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Abstract

An evaluation of high-throughput Fourier-transform infrared spectroscopy (FT-IR) as a technology that could support a “metabonomics” component in toxicological studies of drug candidates is presented. The hypothesis tested in this study was that FT-IR had sufficient resolving power to discriminate between urine collected from control rat populations and rats subjected to treatment with a potent inflammatory agent, bacterial lipopolysaccharide (LPS). It was also hypothesized that co-administration of LPS with ranitidine, a drug associated with reports of idiosyncratic susceptibility, would induce hepatotoxicity in rats and that this could be detected non-invasively by an FT-IR-based metabonomics approach. The co-administration of LPS with “idiosyncratic” drugs represents an attempt to develop a predictive model of idiosyncratic toxicity and FT-IR is used herein to support characterization of this model. FT-IR spectra are high dimensional and the use of genetic programming to identify spectral sub-regions that most contribute to discrimination is demonstrated. FT-IR is rapid, reagentless, highly reproducible and inexpensive. Results from this pilot study indicate it could be extended to routine applications in toxicology and to supporting characterization of a new animal model for idiosyncratic susceptibility.

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1. Introduction

Metabonomics is increasingly utilized in toxicological evaluations of candidate drugs and offers numer-

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ous advantages over conventional histopathological or enzymatic protocols (Nicholson et al., 1999, 2002). 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). Operationally, it involves systematic spectral analysis of biofluid (typically urine) composition in order to associate organ toxicity with specific spectral patterns and to identify surrogate markers of toxicity.

The discipline has seen increasing acceptance in the pharmaceutical industry as evidenced by the formation of Consortium on Metabonomics in Toxicology (COMET) (Breau and Cantor, 2003) and is exemplified by a proven track record of biomarker discovery and early diagnosis of toxicological indications (Nicholls et al., 2000). The measurement technology adopted by COMET is nuclear magnetic resonance (NMR). NMR has considerable merit in such an approach as it is non-invasive, reasonably rapid (<5 min is typical), quantitative, and highly reproducible. This reproducibility allows the development of a stable database that can facilitate comparative assessments of the effect of different drug candidates on urinary metabolic profiles. Metabonomic investigations are now being extended into the phenotyping of transgenic animal models (Nicholson et al., 2002; Gavaghan et al., 2000) and clinical investigations (Brindle et al., 2002).

The increasing interest in extending metabonomics applications has coincided with a concomitant interest in pursuing alternative measurement technologies as complementary options to NMR. Thus, electrospray mass spectrometry (MS), either in direct injection mode (Allen et al., 2003; Goodacre et al., 2002; Vaidyanathan et al., 2001, 2002) or coupled to liquid chromatography (Pham-Tuan et al., 2003; Plumb et al., 2002) has attracted increasing attention. The successful application of electrospray MS coupled to HPLC has now been described for a metabonomic analysis of rat urine from a toxicological study (Plumb et al., 2002).

We were interested in the application of Fourier-transform infrared spectroscopy (FT-IR) in metabonomics since it offers potential advantages in cost, simplicity and low sample volume requirements. Typical acquisition times are 5–10 s per sample.

Furthermore, high-throughput instruments interfaced with a motorized stage that allows sampling from 96-well plates are now commercially available.

FT-IR offers considerable potential in contributing to biomedical studies (Petrich, 2001; Naumann, 2001; Ellis et al., 2003). Its application outside of cancer diagnostics is still limited, but recent literature examples of the use of FT-IR in biomedical research include the analyses of body fluids from diabetes (Petrich et al., 2000) and arthritis patients (Eysel et al., 1997; Staib et al., 2001) brain material infected with transmissible spongiform encephalopathies (Kneipp et al., 2002) and follicular fluid for investigating oocyte development (Thomas et al., 2000). Reports on applications to toxicology remain few but include evaluations of the effects of toxic agents such as carrageenan (Perromat et al., 2001; Melin et al., 2001) and carbon tetrachloride (Melin et al., 2000, 2001) on internal organs. FT-IR analyses of urine have focused primarily on multi-analyte measurements of specific urinary components (Heise et al., 2001) although there is one report on the use of FT-IR-based urine analysis to distinguish normal from rejecting renal allografts (Somorjai et al., 2002). We report now on a pilot study into the suitability of FT-IR as a technology that could be employed in metabonomic investigations in toxicology and on its contribution to characterizing a new model for idiosyncratic susceptibility. This model is based on the concept that mild inflammation induced by bacterial lipopolysaccharide (LPS) enhances the hepatotoxicity of pharmaceuticals and other xenobiotics (Ganey and Roth, 2001). Preliminary publication on this model with ranitidine describes it in more detail (Luyendyk et al., *in press*).

In our pilot study, 29 rats were fasted for 24 h pre-dose. Urine was collected at 6, 18, and 24 h. For the purposes of developing a model for idiosyncratic susceptibility (Ganey and Roth, 2001; Luyendyk et al., *in press*) sets of rats were treated with LPS, ranitidine, and with ranitidine/LPS co-administrations. The premise of the idiosyncratic susceptibility model is that LPS, a potent inflammatory agent, would act as a “sensitizer” to adverse side effects and thereby uncover toxicological responses to drug candidates. Ranitidine (Vial et al., 1991) a H₂ receptor antagonist used in the treatment of gastroesophageal reflux, has been associated with episodes of idiosyncratic hepatotoxicity.

For the purposes of our pilot study, we were focused on determining whether high-throughput FT-IR could discriminate (i) the three pre-dose sample populations (as a first assessment of the discriminating power of FT-IR-based urine analysis), (ii) inflammatory effects of LPS from control vehicle treatments, and (iii) ranitidine-treated subjects from subjects where LPS was co-administered with ranitidine. Our goal was to evaluate FT-IR as a potential metabonomic tool and to assess whether it had the potential to contribute to toxicological studies and to support characterization of the idiosyncratic susceptibility model.

2. Materials and methods

2.1. Animals

Male, Sprague-Dawley rats (CrI:CD (SD)IGS BR; Charles River, Portage, MI) weighing 250–300 g were housed individually in plastic metabolism cages for these studies. Animals were fed powdered certified rat diet (Labdiet 5002, Purina Mills Inc., St. Louis, MO) and allowed access to water ad libitum. They were allowed to acclimate for 5 days in standard group rat laboratory cages and for 2 days in metabolism cages in a 12-h light/dark cycle prior to treatment.

2.2. Experimental protocol

Rats fasted for 24 h were given 44.4×10^6 endotoxin units/kg LPS, (*Escherichia coli* serotype O55:B5, Sigma-Aldrich, St. Louis, MO), or its saline vehicle, intravenously. Two hours later, 30 mg/kg ranitidine or sterile phosphate-buffered saline (PBS) vehicle was administered intravenously. Ranitidine solution was administered at 2 ml/kg at a rate of approximately 0.15 ml/min. Urine from each rat was collected over ice with the addition of sodium azide (0.1% (w/v) urine). Samples were collected at 6, 18 and 24 h during fasting (pre-treatment) and 6, 18, and 24 h after ranitidine 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. The hepatic toxicological effects of these treatments are published elsewhere (Luyendyk et al., in press) but in summary were as follows: vehicle, no injury; LPS, modest inflammation but little to no injury;

ranitidine, no injury; ranitidine plus LPS, moderate to severe inflammation and injury.

2.3. FT-IR analysis

Aliquots (2 μl) of the urine samples were evenly applied onto a zinc selenide microplate containing 96 wells and oven-dried at 50°C for 30 min (or until visibly dry). Aliquots were sampled in triplicate. The FT-IR instrument was a Bruker Vector 33 FT-IR spectrometer (Bruker Optics, Billerica, MA) with a HTS-XT microplate extension, equipped with a DTGS (deuterated triglycine sulphate) detector. Spectra were collected over the wavenumber range $4000\text{--}600\text{ cm}^{-1}$ at a rate of 20 s^{-1} . The spectral resolution used was 4 cm^{-1} . To improve the signal-to-noise ratio, 256 spectra were co-added and averaged. Each sample was thus represented by a spectrum containing 882 points and spectra were displayed in terms of absorbance. Spectra were first normalized so that the smallest absorbance was set to 0 and the highest to +2 for each spectrum. The first and second derivatives of the FT-IR spectra were smoothed with the Savitzky–Golay algorithm (Savitzky and Golay, 1964) using nine-point smoothing. Chemometric analyses were undertaken on the second derivative data.

2.4. Chemometric data processing methods

2.4.1. Principal components analysis (PCA)

Principal components analysis (PCA) was performed via the NIPALS algorithm (Wold, 1966) on the FT-IR 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 (Jolliffe, 1986; Causton, 1987).

2.4.2. Discriminant function analysis (DFA)

Discriminant function analysis (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 *a priori* groups (herein the groups were defined as instrument replicates). In this study, the first 5–20 PC scores were used as the data source for PC-DFA.

2.4.3. Hierarchical cluster analysis (HCA)

The Euclidean distance between *a priori* group centers in PC-DFA space was used to construct a similarity measure, and these distance measures were then processed by an agglomerative clustering algorithm to construct a dendrogram (Manly, 1994).

2.4.4. Selection of discriminatory wavenumbers by genetic programming (GP)

It is also possible to use PC-DFA on a spectral sub-regions chosen by a genetic algorithm (Broadhurst et al., 1997) or by genetic programming (GP) (Gilbert et al., 1997; Goodacre et al., 2000; Taylor et al., 1998). In this study, wavenumbers were selected by GP using the program GMax-BIO (Aber Genomic Computing, Aberystwyth, UK) (Kell et al., 2001) using the default parameter settings proposed by the manufacturer.

3. Results and discussion

Urine collected at three different time-points from the pre-dose rat population represented an opportunity to assess the discriminatory potential use of high-throughput FT-IR in evaluating closely related populations. It offered a much larger data set ($n = 29$) than that for the drug and LPS treated subsets ($n = 5$). Discriminant function analysis (Fig. 1) showed good separation of all three pre-dose urine collection periods and clearly boded well for our investigations into the drug and LPS treated subsets. The data also represent the first time FT-IR has been used to generate metabolic fingerprints of fasting populations.

It is also possible to use PC-DFA on spectral sub-regions or on a selected number of wavenumbers chosen by GP (Gilbert et al., 1997; Goodacre et al., 2000; Taylor et al., 1998). It was clear from our FT-IR data that selected regions of the urine spectra could discriminate the fasting pre-dose populations. The most relevant regions included (but were not limited to) $1276\text{--}1307\text{ cm}^{-1}$ (OH alcohols, CN amides), $1384\text{--}1407\text{ cm}^{-1}$ (OH alcohols, CH aldehyde, alkanes) and $1519\text{--}1542\text{ cm}^{-1}$ (CNH amides, aromatic ring). By combining only the above regions (thus reducing the data from 882 recorded wavenumbers to only 23) PC-DFA offered the same effective separation as seen when the total spectrum was used (data not shown.). Indeed, individual wavenumbers from

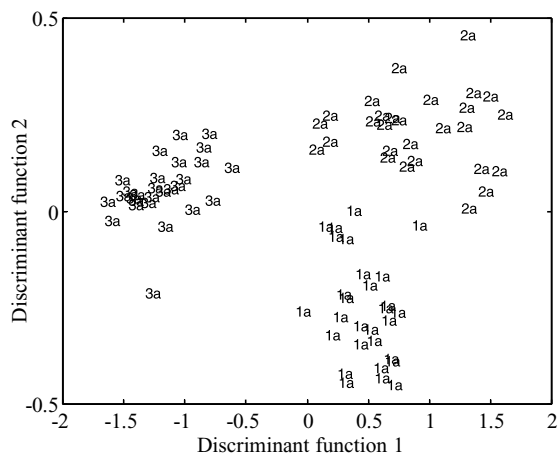


Fig. 1. Discriminant function analysis of urine sampled at three different time-points during the pre-dosing fast; 1a = 6 h ($n = 29$), 2a = 18 h ($n = 28$), 3a = 24 h ($n = 28$). PCs 1–10 were used to construct this PC-DFA plot and these explained 97.8% of the total variance. The number of *a priori* classes used for 1a was 29, 2a was 28, and 3a was 28.

these aforementioned regions could discriminate the 1a and 3a populations on their own (data not shown). Constructing the PC-DFA model on a selected number of more discriminatory inputs, rather than utilizing the entire spectrum, increases the robustness of the PC-DFA model generated. This is recognized as the parsimony principle (Seasholtz and Kowalski, 1993). There is also a supposition on our part that specific patterns of selected variables may be associated with specific toxicological lesions, a hypothesis we hope to evaluate in further toxicological studies.

Despite this initial demonstration that three closely related populations could be discriminated by FT-IR analysis and that chemometric interrogation could discern spectral regions critical to discrimination, to prove of value in metabonomics FT-IR must be able to discriminate the effects of toxic agents from control vehicle treatments. Fig. 2 shows that FT-IR analysis of urine can readily distinguish LPS (i.e. rats with modest hepatic inflammation (Luyendyk et al., in press)) from vehicle treatments. That the controls and treated populations are clearly separated in the first PC-DF indicates that, despite the multiple classes ($n = 5$) used for each population, the two groups (LPS and control) are clearly delineated. Moreover, it is also evident from this figure that there appears to be greater

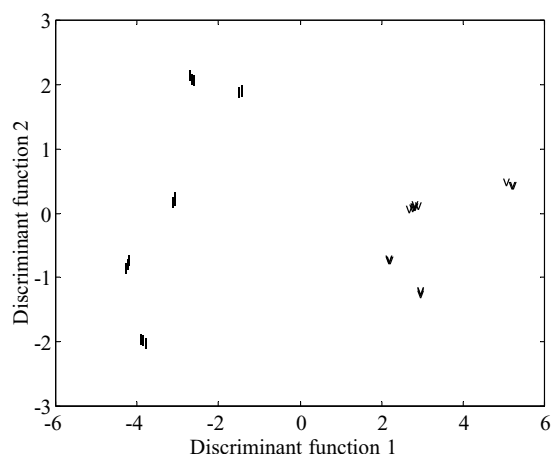


Fig. 2. Discriminant function analysis of urine from LPS (I) treated and control vehicle (v) treated populations. The urine was sampled at 24 h after administration of LPS or vehicle (see Section 2). PCs 1–12 were used to construct this PC-DFA plot and these explained 99.8% of the total variance. Five *a priori* classes were used in each population and these instrumental replicate ($n = 3$) values are shown.

heterogeneity in the LPS-treated populations (I) compared to the controls (v). Fig. 3 shows a cluster plot demonstrating differential clustering of LPS treated, ranitidine treated and ranitidine/LPS treated. As can

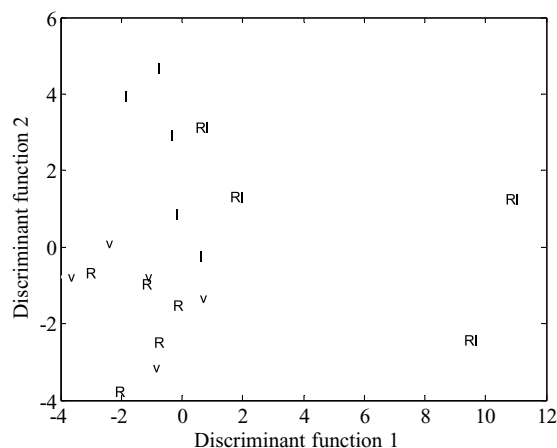


Fig. 3. Discriminant function analysis of urine from LPS (I) treated ($n = 5$), control vehicle (v) treated ($n = 5$), ranitidine (R) ($n = 5$) and ranitidine/LPS (RI) ($n = 4$) treated populations. The urine was sampled at 24 h after administration of test material (see Section 2). PCs 1–20 were used to construct this PC-DFA plot and these explained 99.9% of the total variance. The average of the instrument replicates is shown.

be observed, the largest difference is seen in the first discriminant function which separates ranitidine/LPS treated animals from all others. In other words, those animals treated with a potent inflammatory agent and exposed to ranitidine have very different metabolic fingerprints. The segregation, however, is more apparent in the dendrogram (Fig. 4). This appears to reveal four major clusters but following the branches of the dendrogram to its smallest units reveals no discrimination between vehicle (v) and ranitidine (R) treatments. Therefore, by-and-large, three clusters are recovered: (i) controls and ranitidine treated, (ii) LPS treated and (iii) ranitidine/LPS treated.

To show further the power of FT-IR within this metabolomics setting and to test the cluster analysis model, we removed one of the biological replicates randomly from each of the four treatments prior to cluster analysis. The biological replicate of each of the four classes studied was projected into the resultant PC-DFA model created from the rest of the data (42 spectra; 14 samples analyzed in triplicate), as previously reported. Following this a dendrogram was constructed, as described above, using the combined DFA space from the creation and test results. The dendrogram produced is shown in Fig. 5 and it is clear that the test spectra (those that had been projected, marked with an asterisk) are recovered in their “correct” clusters. This clearly shows that FT-IR has utility in metabolomics.

As was the case in the pre-dose populations spectral sub-regions could be selected to discriminate the compound-treated populations. Interestingly, some of the wavenumbers used in separating the pre-dose populations could separate vehicle from LPS (e.g. 1569–1581 cm^{-1}) and ranitidine from ranitidine/LPS (1519–1542 cm^{-1}) treated subjects. Indeed, when only the original 23 wavenumbers were utilized in PC-DFA good separation between the four post-populations was observed (Fig. 6).

It was also evident from chemometric analysis that the ranitidine and ranitidine/LPS treated populations could be discriminated utilizing only a spectral sub-region ranging from 1735 to 1755 cm^{-1} (C=O). A PC-DFA plot using this additional region to the 23 selected wavenumbers described above gave good discrimination (data not shown).

NMR analyses of the same urine samples assayed above showed increases in creatinine, creatine,

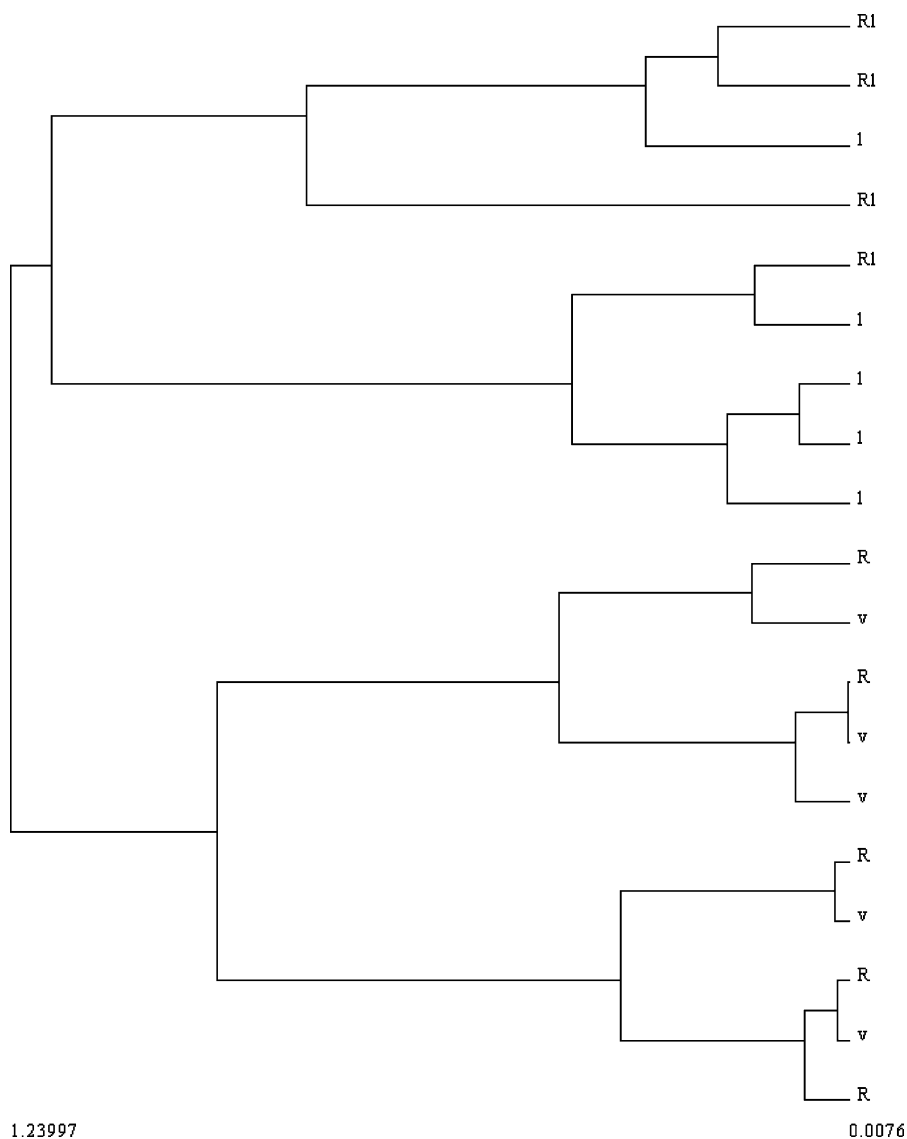


Fig. 4. Hierarchical cluster analysis of urine from LPS (1) treated, control vehicle (v) treated, ranitidine (R) and ranitidine/LPS (RI) populations shown as a dendrogram. The urine was sampled at 24 h after administration of test material see Section 2).

taurine, and acetate and decreases in hippurate, citrate and 2-oxoglutarate on exposure to ranitidine/LPS co-administrations (Maddox et al., in preparation). Using FT-IR spectral observations to assess concentration changes in urinary metabolites is more challenging since the spectra essentially represent a signature of all metabolites.

The FT-IR findings from the above experiments were consistent with histological findings. Those subjects treated with the co-administration regimen showed greater hepatic lesions, inflammation and cell death (Luyendyk et al., in press). The fact that we could discriminate these populations and identify spectral regions critical to discrimination validates

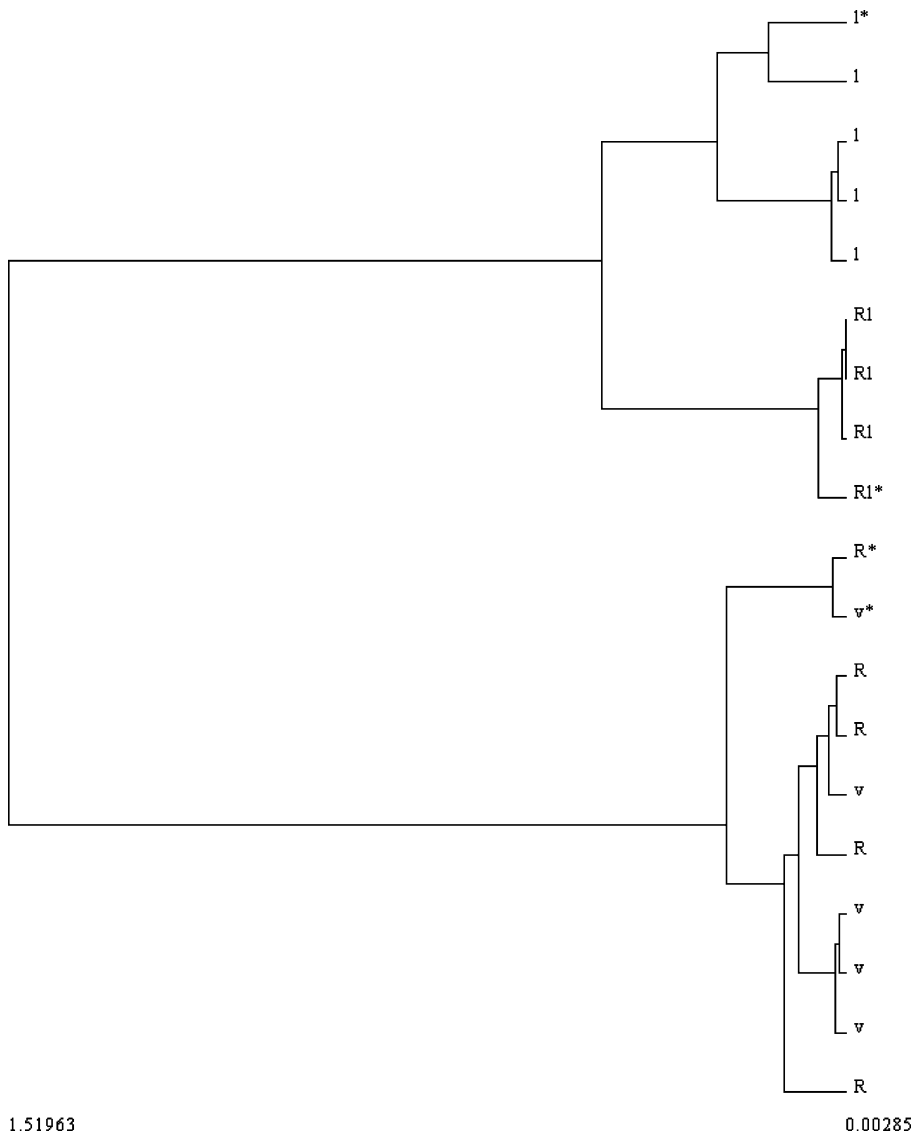


Fig. 5. Validated hierarchical cluster analysis of urine from LPS (l) treated, control vehicle (v) treated, ranitidine (R) and ranitidine/LPS (R1) populations shown as a dendrogram. The urine was sampled at 24 h after administration of test material (see Section 2). The samples marked with an asterisks were projected into PC-DFA space prior to HCA.

application of FT-IR as a tool in toxicological studies. We therefore conclude that FT-IR could be of value in routine toxicological studies and has already contributed to characterization of a model for idiosyncratic toxicity.

FT-IR is rapid but chemically unselective and is therefore appropriate for fingerprinting approaches that do not necessarily require identification of specific

targeted metabolites of interest. Indeed, it has become apparent from NMR-based metabonomics that single metabolites are rarely specific to a given pathophysiological state. For example renal papillary damage can be revealed by specific permutations of changes in urinary levels of trimethylamine-*N*-oxide, dimethylamine, acetate and succinate (Holmes et al., 1997). None of these markers on their own have diagnostic

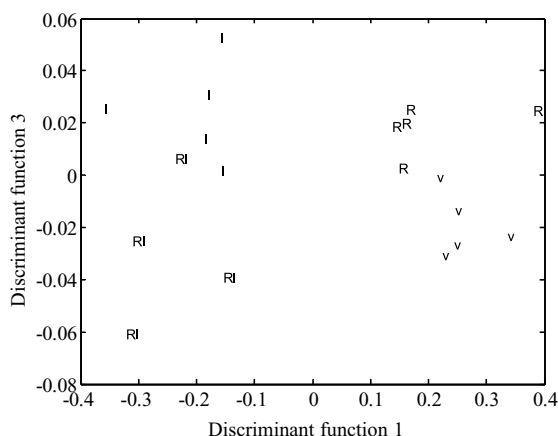


Fig. 6. Discriminant function analysis of urine from LPS (I) treated, control vehicle (v) treated, ranitidine (R) and ranitidine/LPS (RI) populations using 23 pre-selected wavenumbers as in Fig. 2. The urine was sampled at 24 h after administration of test material (see Section 2). PCs 1–5 were used to construct this PC-DFA plot and these explained 98.2% of the total variance. The average of the instrument replicates is shown.

value. The biomarker information achievable through FT-IR is considerable, as recorded spectra will represent a signature of all components present in urine.

The NMR-based metabolomics enterprise has benefited from many years of excellent and well-considered research and evaluation of numerous toxins known to induce specific pathological lesions. No such systematic applications in FT-IR are apparent from the literature. The low expense and rapidity of FT-IR and our own initial findings suggest that this may prove to be a worthwhile endeavor. The preliminary data on LPS modulated toxicity also suggest further efforts in supporting a model for idiosyncratic susceptibility are warranted.

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