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Short Research Article

Isotopic enrichment enhancement in metabonomic analysis of UPLC–MS data sets[†]

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Abstract: Isotopic enrichment of compounds dosed in drug metabolism studies greatly enhances the spectroscopic differentiation of xenobiotic and endogenous metabolites. We show the application of $^{13}C^{15}N$ labelling of a model drug (4-cyanoaniline) as an aid to metabonomic studies of UPLC–MS-generated metabolic data sets. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: drug metabolism; isotope labelling; substituted anilines; metabonomics; metabolomics

Introduction

Metabonomics[1,2] is an approach for investigating system-level, time-resolved metabolic profiles generated *in vivo*. Metabonomic techniques (the chemometric interrogation of spectroscopically derived data sets) have been successfully applied in numerous areas of bioscience including drug discovery and toxicity,[3] disease diagnosis,[4] infection characterization,[5] functional genomics,[6] and epidemiological research.[7] The integration of metabonomics with other ‘omics’ sciences has led to systems biology approaches that promise to provide personalized healthcare incorporating both genetic and environmental factors.[8] Metabonomic profiling may be done on numerous analytical platforms, the most common being high-resolution nuclear magnetic resonance (NMR) spectroscopy[9] and liquid chromatography–mass spectrometry (LC–MS).[10] The complementarity of these techniques has recently been harnessed to provide integrated LC–NMR–MS metabonomic analyses through the use of Statistical HeterospectroscopY (SHY).[11]

In metabonomic or metabolomic classification of biological samples from animals or cells treated with drugs, it is crucial to separate classes based on endogenous metabolites only so that biomarker recovery can proceed efficiently. The widely practiced approach of isotope labelling for metabolism studies[12] now finds new relevance as it can also be applied as an editing tool to separate xenobiotics from endogenous compounds. We present here, an approach using stable isotope label incorporation to a simple substituted aniline as a metabonomic enhancement tool. This forms part of an ongoing series of investigations into the metabolism of aromatic amines as drug models.[13–18] Aromatic amines undergo extensive metabolism *in vivo* and exhibit a wide range of associated toxicities.[19] The mechanisms that underlie their metabolism and toxicity are of interest and recent attempts to predict metabolic and toxicological outcomes have used anilines as model compounds.[20] Additionally, efforts to predict the metabolic fate of substituted anilines and investigate the physicochemical properties that govern their behaviour *in vivo* have been made using computational and multivariate analysis methods.[21] In this work, we have focused on evaluating the utility of a stable label in the context of metabonomic data set analysis to differentiate efficiently xenobiotic and endogenous metabolites.

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Methods

In the present work, a 50:50 mixture of [^{12}C, ^{14}N] 4-cyanoaniline and [^{13}C, ^{15}N]4-cyanoaniline was administered as an intraperitoneal dose at 50 mg kg^{-1} to bile cannulated, Wistar-derived rats. The latter (dual labelled) compound was prepared in such a way that both the carbon and nitrogen isotopes were situated in the cyano-group. This label was used to produce pairs of peaks in mass spectra with equal intensity, separated by two m/z units when collected biofluids were analysed by ultra-performance liquid chromatography–MS (UPLC–MS).[22] Following surgery under anaesthetic to insert a cannula into the bile duct for bile collection, animals were allowed to acclimatize in individual metabolism cages for 72 h prior to dosing. Food (R&M no. 1 modified irradiated diet) and water were available *ad libitum*. Twelve hours dark/light cycles were maintained throughout the study. Urine samples were collected over dry ice in the periods 0–24 and 24–48 h post dose. Bile samples were collected via the cannula over dry ice in the periods 0–6, 6–12, 12–24 and 24–48 h. Urine samples used for UPLC–MS profiling were diluted 4-fold with water and centrifuged (13 000 rpm, 10 min). A 20 μL injection was used.

Chromatographic separations were performed on a 2.1 mm × 100 mm Acquity UPLC™ BEH C_{18} column (1.7 μm particle size), using an Waters® Acquity UPLC™ system (Waters, Watford, UK) comprising a binary solvent manager, automatic sample handler, column oven (40°C), and 2996 PDA detector, and managed using Empower software. Eluent flow was split using a zero-dead-volume T-piece, with 25% of the flow (0.15 mL min^{-1}) to the mass spectrometer, and 75% (0.45 mL min^{-1}) to waste. A gradient elution of water (solvent A) and acetonitrile (solvent B) was used to facilitate the separation: 0–20%B over 4 min, 20–95%B from 4 to 9 min.

MarkerLynx™ software (Waters, Milford, MA, USA) was used to identify and tabulate retention time-m/z pairs of peaks detected in the UPLC–MS data using a proprietary algorithm. These data were exported to Simca P+ 11 (Umetrics, Umea, Sweden) and mean-centred prior to multivariate analysis. Pattern recognition by PCA[23,24] was used to visualize the latent structure in the data set and identify metabolites responsible for any observed discrimination between predose and post dose samples. Signals arising from compound-related material were rapidly identified as isotopic clusters in mass spectra and by conspicuous pairs of variables in the PCA loadings plots. These were identified as metabolites of 4-cyanoaniline and the effect of removing these signals on the latent structure in the data was investigated.

Results and discussion

Following UPLC–MS profiling of urine and bile collected from rats dosed intraperitoneally with a 50:50 mixture of [^{12}C,^{14}N]4-cyanoaniline and [^{13}C,^{15}N]4-cyanoaniline there were noticeable differences in the base peak intensity (BPI) traces, as illustrated in the case of urine in Figure 1.

The 'Strip' function incorporated in MassLynx™ allowed the extraction of all signals in the data set that appeared as a 'doublet' of equal intensity, with m/z difference of 2 units, giving rise to a 'xenochromatogram'. An example is shown in Figure 1. In

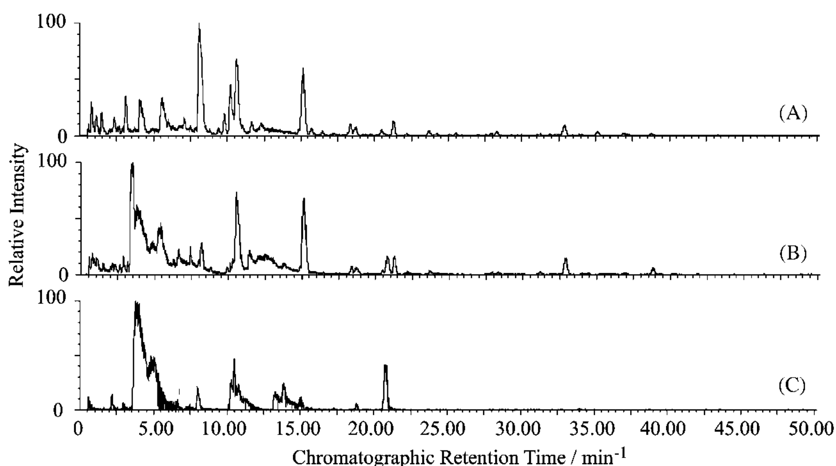

Figure 1 Negative ion mode UPLC–MS BPI trace of urine samples collected predose (A) and during the period 0–24 h (B) following intraperitoneal administration of isotopically labelled 4-cyanoaniline to Wistar-derived rat. (C) 'Stripped' trace of 0–24 h urine sample consisting of only those signals that satisfied the designated isotopic criteria (1:1 intensity ratio (±10%) and m/z difference of 2 (±0.1 units)).

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addition to being useful in guiding metabolite profiling and identification, such traces may also find a use in chromatographic assay optimization, e.g. the optimization of runtime/solvent use or maximizing the chromatographic dispersion of metabolites. The vast majority of these signals were attributed to metabolites of 4-cyanoaniline as seen in previously published work on the metabolism of cyanoanilines.[25] In this respect, the incorporation of a stable label greatly increased the speed with which metabolites and associated fragment ions could be identified. The main route of metabolism was found to be ring hydroxylation and sulfate conjugation. The products of numerous other biotransformations including *N*-acetyl and *N*-acetylcysteine conjugates were also detected.

The same UPLC–MS data set was investigated using simple chemometric methods to demonstrate the utility of incorporating a stable label in a dosed xenobiotic to aid metabonomic analyses. MarkerLynx™ identified 137 retention time – m/z markers using a proprietary peak picking algorithm. A principal component analysis (PCA) model with two components was generated ($R^2X = 0.73$). The resulting PCA scores plot (Figure 2(A)) shows a separation of the predose and post dose samples. From the corresponding PCA loadings plot (Figure 2(B)), it can be seen that there are several variables that are markedly increased in the post dose spectra, and that many of these appear as pairs of variables on the plot. Close inspection of these variables showed that a number could be easily identified as being metabolites of 4-cyanoaniline (identical retention time, two m/z units apart). The proximity of variables in the loadings plot also indicates that the peaks they represent are of similar intensity, as no scaling was applied to the data set.

The exclusion of pairs of variables corresponding to metabolites of 4-cyanoaniline (leaving only variables relating to endogenous compounds), and subsequent regeneration of a two-component PCA model ($R^2X = 0.755$), gave rise to the scores and loadings plots shown in Figure 3. The degree of separation between the predose and post dose samples in the

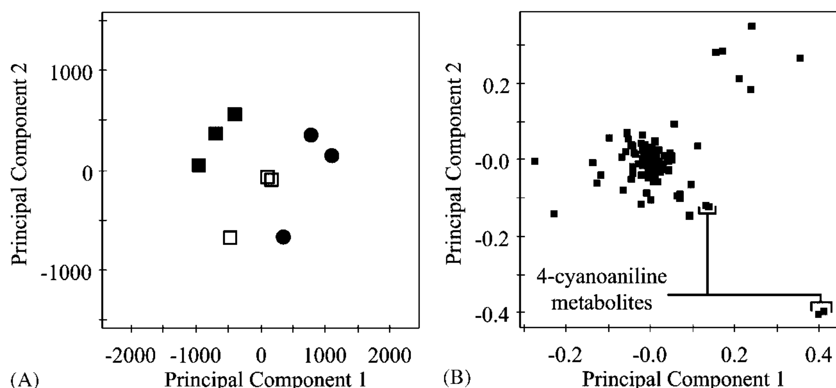

Figure 2 Visualization of multivariate models generated from UPLC–MS data of 4-cyanoaniline profiling: PCA scores plot (A) solid square: predose urine, circle: 0–24 h urine, hollow square: 24–48 h urine; PCA loadings plot (B) each point corresponds to one retention time – m/z marker selected by MarkerLynx™.

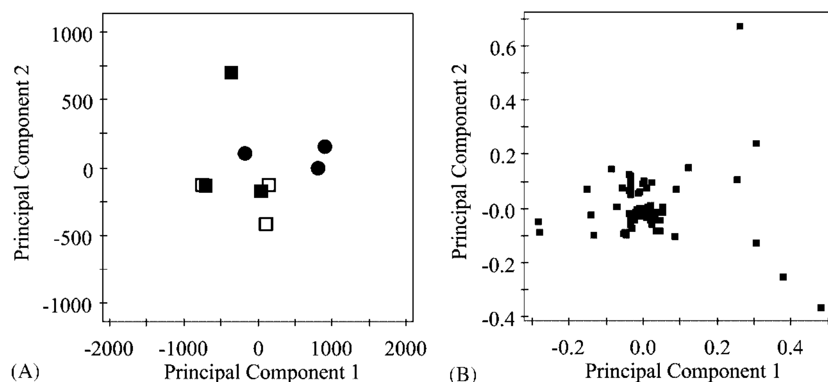

Figure 3 Visualization of multivariate models generated from UPLC–MS data in this study following removal of 4-cyanoaniline metabolites. PCA scores plot (A) solid square: predose urine, circle: 0–24 h urine, hollow square: 24–48 h urine; PCA loadings plot (B) each point corresponds to one retention time – m/z marker selected by MarkerLynx™.

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projection is clearly less well defined than prior to this process (Figure 2(A)), indicating that xenobiotic metabolites were making a substantial contribution to this separation.

Xenobiotic metabolism studies are typically conducted on the minimum possible number of animals. Ethical considerations concerning the refinement, reduction and replacement of experimental animals are of increasing importance, and often the use of large numbers of animals cannot be easily justified. The primary aim of the present study was to investigate the metabolism of a substituted aniline and the combination of a stable isotope in the dosed compound, combined with chemometric pattern recognition as a data analysis tool. Such an approach may be suitable for certain applications in drug discovery.

The small sample set in this study does not lend itself to robust metabonomic analysis that would reveal complex, time-resolved changes in endogenous metabolites. However, it has been shown that the sequential removal of pairs of variables relating to xenobiotic metabolites – aided by isotopic enrichment – and regeneration of a multivariate model eventually yields a data set containing only signals of endogenous origin. Such a data set would be ideally suited to metabonomic analysis, given a larger cohort of animals/samples.

Conclusion

These studies have illustrated that stable isotope labels can be used to enhance the utility of LC–MS data in metabolism studies. This combination of a stable isotope label, ultra-performance liquid chromatography-MS (UPLC–MS) and chemometrics – as demonstrated here as a proof-of-principle – may be of interest to those in the field of metabonomics. Efficiently differentiating spectrometric signals of endogenous and xenobiotic origin may help reduce the opportunity for incorrect assignment of xenobiotic metabolites as potential biomarkers of response requiring characterization.

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