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Journal of Chromatography B, 817 (2005) 67–76
JOURNAL OF CHROMATOGRAPHY B
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Review

HPLC-MS-based methods for the study of metabonomics

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Received 10 March 2004; accepted 20 July 2004
Available online 25 September 2004

Abstract

The development and use of HPLC-MS for the study of metabonomics is reviewed. To date the technique has been applied to the analysis of urine samples obtained from studies in rodents in investigations of physiological variation (e.g., factors such as strain, gender, diurnal variation, etc.) and toxicity. Examples are provided of the use of conventional HPLC, capillary methods and the recently introduced high-resolution systems based on a combination of high pressure and small particle size ("UPLC"). Comparison is also made of the use of ^{1}H NMR spectroscopy and HPLC-MS for the analysis of biofluid samples and the advantages and limitations of the two approaches are assessed. Likely future developments are considered.

Keywords: Metabolite profiling; Metabonomics; Metabolomics; HPLC-MS; Capillary HPLC-MS; High-resolution HPLC-MS; UPLC

Contents

1. Introduction

Metabonomics [1] is a term used to describe the non-targeted "global" analysis of tissues and biofluids for low molecular mass organic endogenous metabolites. Using the metabolic fingerprints, thus obtained, it is often possible to distinguish between strains of animal, disease states or to detect pharmacological or toxic effects obtained following the administration of, e.g., candidate drugs. In combination with genomics, transcriptomics and proteomics, metabonomic analysis is being increasingly used in the discovery and development of new medicines. Much, therefore, depends on the ability of the analytical technique employed to detect often-subtle differences in the complex mixtures found for biofluids, such as urine and plasma, or in tissue extracts. Con-

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doi:10.1016/j.jchromb.2004.07.045

ventionally, much work in metabonomics has been performed using high field nuclear magnetic resonance (NMR) spectroscopy (e.g., see references [2–6]). In this type of application, NMR has many advantages, not the least of which are the high information content of the resulting spectra, the relative stability of NMR-chemical shifts, the ease of quantification and the lack of any need to pre-select the conditions employed for the analysis. This contrasts with most chromatographic methods where the need to select columns and elution conditions may result in an unintended bias to the analysis plus the potential for retention times to drift making comparison between runs potentially more difficult. Whilst GC–MS-based analysis has been employed for metabolomic analysis for plant and microbial metabolite fingerprinting [7–10] together with a number of publications describing HPLC-MS [11,12] or CE–MS-based techniques [13] the use of chromatographic techniques for metabonomics has not been widespread. However, recently HPLC-MS has begun to be employed in this area, either alone [14–17], or in combination with NMR spectroscopy [18,19]. This article provides an overview of the use of conventional HPLC-MS for metabonomics and then illustrates the improvements that can result from going to systems with reduced spectral overlap and higher sensitivity such as capillary HPLC. Finally, the advantages of the newly introduced very high-resolution "ultra performance LC" (UPLC) based on sub 2 μm packing materials and high-pressure solvent delivery, in combination with MS, for the analysis of metabonomic samples will be described.

2. Current practice of HPLC-MS in metabonomics

As indicated in Section 1, the application of HPLC-MS for metabonomic studies is relatively new. Such studies, as have been published, include the investigation of toxicity in rats by Plumb et al. [14], Idborg-Bjorkman et al. [15] and Lafaye et al. [16] and metabotyping (metabolic fingerprinting) [20] of strain, gender and diurnal variation in mice Plumb et al. [17].

In our own studies, we have used gradient reversed-phase HPLC-orthogonal acceleration (oa) -time-of-flight (TOF) -MS and MS/MS for the examination of urine from various strains of rat (in preparation) and mouse [17] and from rats exposed to a number of nephrotoxins [18,19]. In these studies, a simple linear gradient has usually been applied with the samples analysed using both +ve and −ve electrospray ionisation (in separate analytical runs). In a typical experiment, a 10 μl aliquot of urine was injected onto a 2.1 mm × 10 cm Symmetry® C18 3.5 μm HPLC column. The column was maintained at 40 °C and elution was performed with a linear gradient of 0.1% aqueous formic acid to 20% acetonitrile (containing 0.1% formic acid) over the period 0.5–4 min. This was followed by an increase in the acetonitrile content to 95% over the period 4–8 min. After holding the solvent composition at 95% acetonitrile for a further minute the column was then returned to its starting conditions. An eluent flow rate of 600 μl /min was used and mass spectrometric data was collected over the mass range of 50–850, in either positive or negative ion mode on a time-of-flight mass spectrometer. The column eluent was split such that approximately 120 μl/min was directed to the mass spectrometer.

As part of our investigations into the utility of HPLC-MS, we elected to study the effects of strain and gender on the urinary metabolic profiles of black, white and nude mice [17]. Samples were collected in both the morning and the afternoon so that we could also examine diurnal variation, as in previous NMR-based studies, differences were readily observed for both strain and collection time [20,21]. Mice represent an important experimental animal for the discovery of new

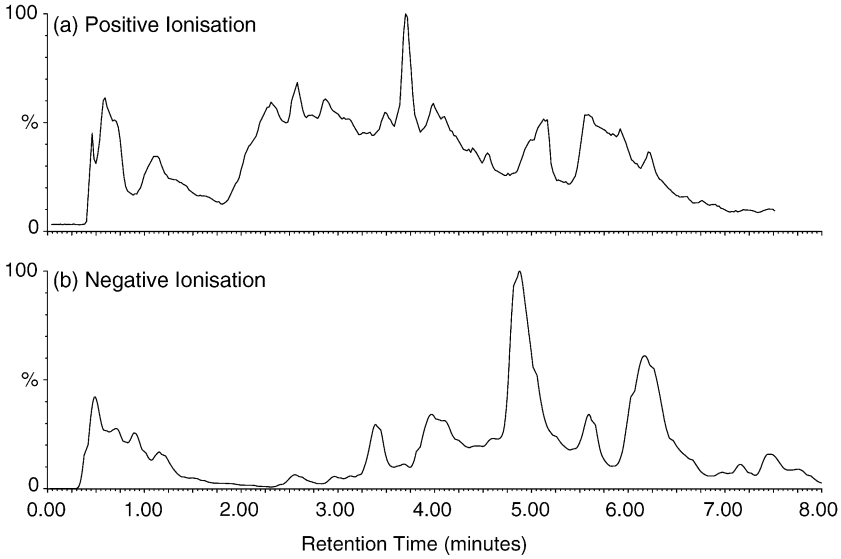

Fig. 1. Representative positive total ion current chromatograms (TIC) obtained in (a) positive and (b) negative ion electrospray for a urine sample collected from a black male mouse in the morning. Visual inspection of the two TIC plots shows that the two modes of ionization will generate different metabonomic information based on the ionizability differences of endogenous components in urine [17].

drugs, with the nude mouse being especially important for the development of anticancer compounds. An understanding of typical "baseline" physiological variation is therefore an essential pre-requisite for in-depth metabonomic studies in drug discovery and development. Typical positive and negative ion total ion current chromatograms (TICs) for a morning sample obtained from a black male animal are shown in Fig. 1. Such profiles contain many hundreds of individual components rendering visual comparison complex. However, by using pattern recognition approaches based on principal components analysis (PCA), it was possible to show that all three strains of mouse could be separated from each other based on their MS-detected metabolite profiles. A typical example of such PCA analysis is shown in Fig. 2, for black, nude and white male mice, where the scores plot resulting from the positive ion HPLC-MS data for the morning sample collection period is shown. One of the ions that contributed to this separation was *m/z* 206.0453, corresponding to $C_{10}H_8NO_4$. When subjected to MS/MS analysis, this ion was provisionally identified, from the product ion spectra and elemental composition, as 4,8 dihydroxyquinoline-2-carboxylic acid, a metabolite found in the tryptophan pathway, and this was confirmed by comparison of the HPLC-MS/MS properties of an authentic standard. Similarly, female animals from the three strains were readily differentiated from each other by PCA, as indeed were male and female animals from all three strains [17].

More recently we have examined urine samples from male and female Zucker rats, a widely used model of insulin resistance, for effects of gender and diurnal variation, with similar results to those seen with mice. A typical example of such a run, illustrating the complexity of the samples, is shown in

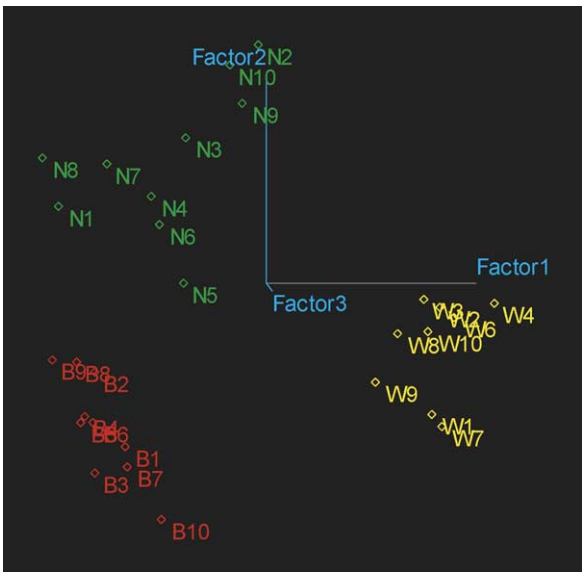

Fig. 2. Resulting scores plot for PCA of black, nude and white male mice for the positive ion HPLC-MS data obtained for the morning sample collection period. Key: green = nude, yellow = white and red = black. Distinct clustering is observed among all three groups according to strain [17].

Fig. 3, where a two-dimensional "map" of *m/z* data versus run time is shown. Over 1500 ions are detectable in the 10 min run illustrating both the power of the technique and the complexity of the samples. It should be noted that not all of these ions may represent individual compounds as some may result from in-source fragmentation or adduct formation.

One of the main applications for NMR-based metabonomics in pharmaceutical research is in the study of toxicology. This has also been an area where HPLC-MS has found

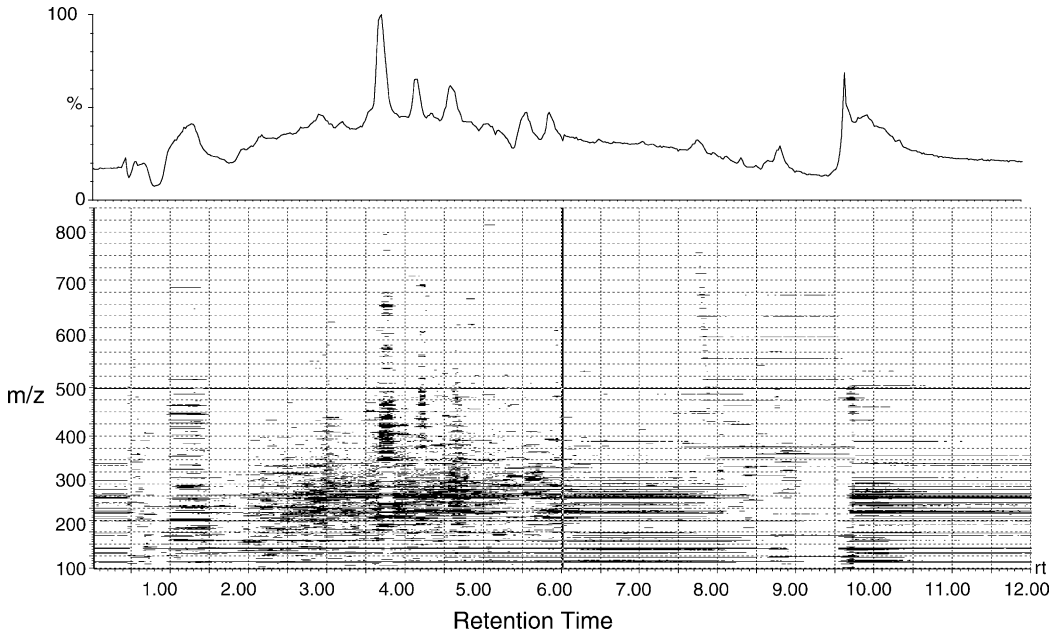

Fig. 3. Gradient HPLC-MS of urine obtained from a male Zucker rat showing both the total ion current mass chromatogram and a 2D mass chromatogram. In this typical chromatogram, over 1500 ions can be detected in 10 min.

early application with three studies published to date. In the first of these studies, Plumb et al. [14] used HPLC-TOF-MS to examine the toxicological profile of an undisclosed candidate drug. Thus, male and female rats were dosed with either dosing vehicle or the candidate pharmaceutical at one of two dose levels (2 and 18 mg/kg) for a period of 3 months. The analytical methodology employed was similar to that used for the mouse studies described above and urine samples were taken at 0–8 and 8–24 h after dosing. Subsequent HPLC-MS and PCA of these urine samples allowed the dose groups to be separated from the control animals and showed both the low-dose and high-dose group samples moving away from the control group on different trajectories. It can be argued that a reason for this is that, whilst the low dose group did not suffer from drug toxicity, there was nevertheless an observable pharmacological effect of the compound on the metabolite profile.

From the loadings plot generated in this study it was possible to identify an ion at *m*/*z* 212 in negative ESI that contributed significantly to the clustering observed within the data. Identification of this metabolite was aided by accurate mass analysis, which gave an elemental composition of $C_8H_6NO_4S$ and a molecular mass of 212.0018 with an accuracy of 4.7 ppm. Further, MS/MS, using a hybrid quadrupole time of flight MS, suggested that this metabolite was indican. This identification was confirmed by comparison with the chromatographic and MS spectral properties of an authentic standard. Indican is a metabolite of tryptophan, which is converted to indole by intestinal bacterial cleavage of the tryptophan side chain.

The separation of control and dosed groups of rats by HPLC-MS using +ve and −ve electrospray ionisation in another toxicity study on the compound citalopram, of interest because of its potential to induce phospholipidosis, was recently reported by Idborg-Bjorkman et al. [15]. The chromatographic separation was performed on an XTerra C18 bonded reversed-phase HPLC column (3.5 μm, 2.1 mm × 150 mm) using a solvent gradient. The solvents were 10 mM ammonium acetate, adjusted to pH 4 with formic acid, and acetonitrile with the starting conditions set at 90% of the aqueous buffer. After a short (2 min) period of isocratic elution, the organic component was increased to 90% over 15 min, followed by a further isocratic elution for 8 min, and then a return to the starting condition where the column was re-equilibrated (6.5 min) prior to the next injection. Prior to HPLC-MS analysis the urine samples were subjected to solid phase extraction (on a retentive polymeric SPE packing). Aliquots of 0.5 ml were applied to pre-activated cartridges (methanol and then 10 mM pH 4 ammonium acetate buffer), and then washed with 0.5 ml of buffer followed by elution with 0.5 ml of methanol. This eluate was then directly injected onto the HPLC and, provided that only 5 μl were applied, no adverse effects on chromatography were seen. Clearly, when global metabolite profiles are required, sample pre-treatment steps such as SPE or liquid–liquid extraction must be carefully optimised to avoid the loss of important analytes. However, such treatments can be used to advantage to eliminate interfering salts, concentrate the samples and provide them in a suitable form for subsequent HPLC-MS analysis. In addition, where there is a need to isolate a particular analyte(s) for subsequent identification methods, such as SPE, can provide a valuable first step.

When the resulting profiles were examined by multivariate data analysis a number of potential markers were observed in the urine samples, though these remained unidentified in this study. As these authors observed, care must be taken with the analysis of the pattern recognition results from studies such as this because the presence of drug metabolites may well cause the data to cluster inappropriately. Indeed, until unequivocally identified all compounds highlighted, as potential biomarkers should be treated with a degree of scepticism. The phenomenon of drug metabolites contributing to the cluster separation has been exploited [22] as an aid to metabolite identification Here, two new drug metabolites were detected, resulting from metabolic cleavage, that would not have been predicted by the usual phase I/II metabolic transformations.

A recent HPLC-MS-based metabonomic study was an investigation of heavy metal toxicity in the rat by Lafaye et al. [16], where urine samples were analysed following the administration of either uranyl nitrate or cadmium chloride. These toxins were added to the drinking water of the test animals at a concentration of 100 mg/l for a period of 3 months. Urine samples were then taken at weekly intervals for the first month and then monthly. Analysis was by reversed-phase gradient HPLC on an XTerra MS C18 bonded column (5 μm, 2.1 mm × 150 mm) at 30 °C. The gradient was formed from 10 mM ammonium acetate (pH 6.7) and methanol beginning at 100% aqueous for the first 10 min rising to 80% methanol over the next 95 min, remaining at this composition for 10 min before returning to the starting conditions. In this case, mass spectrometric analysis was employed using both +ve and −ve ESI, with an ion trap, over a range of 100–1000 amu. Some 30 resolved peaks were detected in +ve and 20 in −ve ESI, respectively. Differences were noted between the two heavy metals toxins in terms of the metabolites seen to vary with dosing, but the bulk of these were unidentified. In this case, examination of the data was performed manually rather than using pattern recognition techniques These authors were able to identify a number of molecules including, e.g., riboflavin, phenol sulphate, ferrulic acid and 7-methylquanine, etc. together with the characterisation of various classes of metabolites (carboxylic acids, alkylamines, sulphates, glucuronides, glycosides, etc.) based on the observation of various characteristic neutral losses.

3. Comparison of HPLC-MS with NMR spectroscopy

As indicated in the introduction, until recently the bulk of metabonomics investigations have been performed using

high field NMR spectroscopy. However, as part of our evaluation of the usefulness of HPLC-MS for metabonomic studies, we have performed a number of studies [18,19] where the results obtained by HPLC-MS have been compared directly with those of high field ^{1}H NMR spectroscopy on the same samples. In the first of these studies the effects of the administration of a single dose of the model nephrotoxin mercuric chloride (2.0 mg/kg, subcutaneous) to male Wistar-derived rats on the urinary metabolite profiles of a range of endogenous metabolites has been investigated [18]. Urine samples were collected daily for 9 days from both dosed and control animals. Analysis of these samples by gradient reversed-phase HPLC on a similar system to that used for the mouse urine profiling study [17] revealed marked changes in the pattern of endogenous metabolites as a result of $HgCl_2$ toxicity. Disturbances in the urinary metabolite profiles were most pronounced (using both NMR spectroscopy and HPLC-MS) at 3 days post dose. Thereafter, the urinary metabolite profile gradually returned to a more normal composition. The PCA analysis of these samples showing control and dosed animals from positive and negative ion MS data is shown in Fig. 4a and b.

Markers of toxicity identified by ^{1}H NMR spectroscopy were raised concentrations of lactate, alanine, acetate, succinate, trimethylamine (TMA) and glucose. Reductions in the urinary excretion of citrate and α-ketoglutarate were also seen. Markers identified by HPLC-MS, in positive ion mode, were kynurenic acid, xanthurenic acid, pantothenic acid and 7-methylguanine, which decreased after dosing. In addition, an ion at *m*/*z* 188, probably 3-amino-2-naphthoic acid, was observed to increase after dosing. As well as these identified compounds other ions at *m*/*z* 297 and 267 decreased after dosing. In negative ion mode a range of sulphated compounds were observed, including phenol sulphate and benzene diol sulphate, which decreased after dosing. As well as the sulphated components an unidentified glucuronide at *m*/*z* 326 was also observed to decrease after dosing. An interesting conclusion from this study was that, whilst both NMR and HPLC-MS showed a similar time course of onset of toxicity and recovery, the markers seen for each technique were quite different suggesting a useful role for both types of analysis. The complementary nature of the two techniques was confirmed in a subsequent study of the nephrotoxicity of the immunosuppressant cyclosporin A [19]. Here, animals were dosed daily with the drug, at 45 mg/(kg day) for 9 days with toxicity only becoming apparent, again by both NMR and HPLC-MS, after 7 days of administration. In this instance, ions resulting from both the drug, its metabolites and the dosing vehicle had to be eliminated from the HPLC-MS data prior to analysis by PCA. This was not a significant problem for the NMR analysis as these components were not present in detectable amounts.

The results from these, and other unpublished studies, clearly indicate the complementary nature of HPLC-MS and NMR spectroscopy for metabonomic investigations and in general we would recommend that, wherever possible, both techniques should be used to analyse samples.

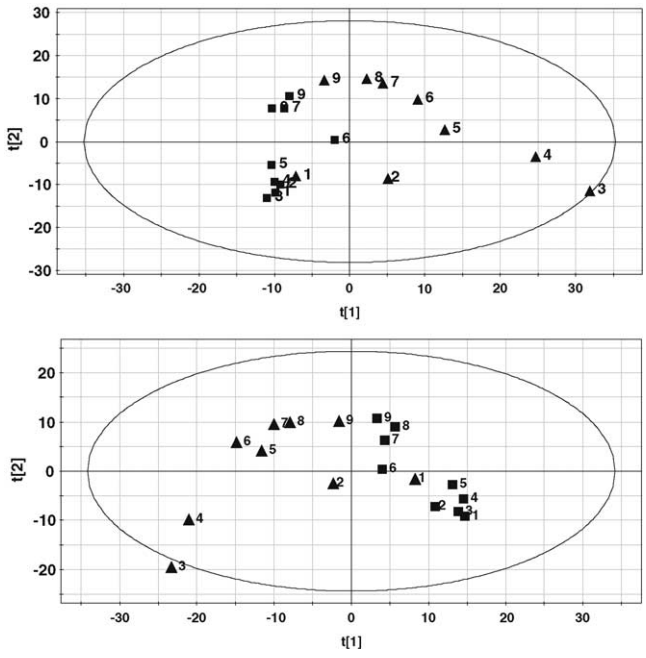

Fig. 4. HPLC-MS positive ion PCA scores plot (upper) and HPLC-MS negative ion PCA scores plot (lower) of the data obtained for a toxicity study on mercuric chloride in the rat [18]. Key: triangles: treated animals; and squares: controls. Numbers next to the symbols represent the day of study (1–9) after dosing.

4. Capillary HPLC-MS for metabonomics

In the case of the Zucker rat example described earlier some 1500 peaks were detectable by conventional HPLC-MS. However, if the gradient were to be extended this number can be significantly increased, albeit at the expense of a longer analysis time per sample (unpublished observations); this is mainly due to the reduction in spectral overlap and the consequent ion suppression effects. Whilst clearly desirable in terms of providing a more comprehensive metabolic fingerprint, such an increase in analysis time is, however, incompatible with the requirements of the analysis of large numbers of samples in a “medium” throughput environment. We were therefore interested in determining whether the use of other HPLC separations had beneficial effects. One such alternative technique where ion suppression in the ion source of the mass spectrometer might be expected to be reduced is capillary HPLC. Indeed this approach has been employed for just this purpose in the plant metabolomics area where home-made C18 bonded monolithic capillary columns (0.2 mm i.d. of 30–90 cm in length) were used with MS detection for *Arapadopsis thaliana* extracts [11].

The system chosen for analysis of the Zucker rat urine samples was based on a 320 μm capillary, 10 cm in length containing a 3.5 μm symmetry C18 alkyl bonded packing. And a 0.2 μl injection of diluted sample was made onto the

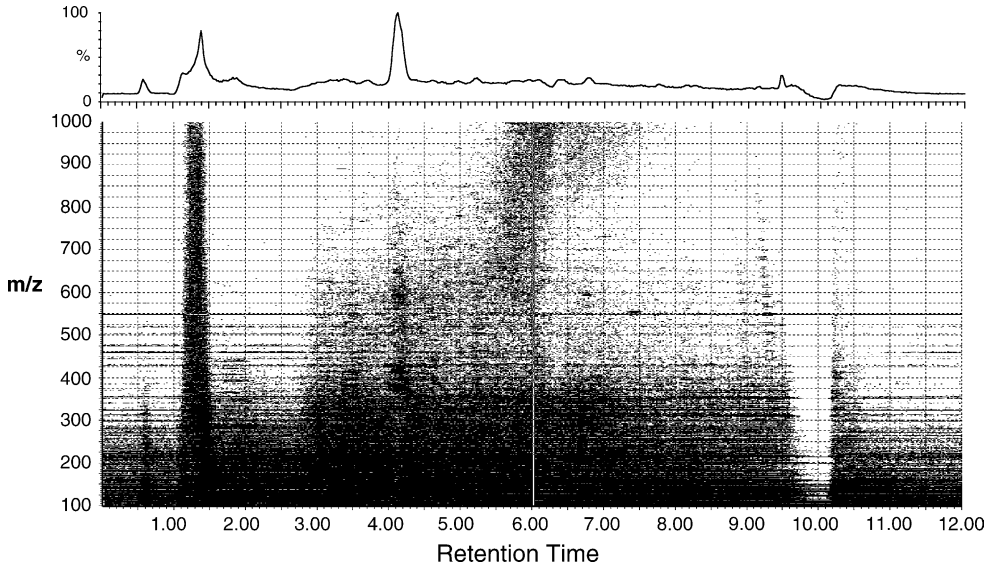

Fig. 5. Capillary HPLC-MS of urine obtained from a male Zucker rat urine with corresponding 2D mass chromatogram. A two-fold increase in detectable peaks is observed in comparison to conventional HPLC-MS.

capillary column. The column was maintained at 40 °C and eluted with a linear gradient of 0–95% acetonitrile containing 0.1% aqueous formic acid versus 0.1% formic acid in acetonitrile using the same gradient profile as for Fig. 3, at 10 μl/min. The results obtained for this system are shown in Fig. 5 and show a significant improvement in the number of peaks detectable with over 3000 putative metabolites present. This increased number of metabolites also enables increased discrimination between, e.g., samples obtained from female animals for a.m. and p.m. collection times. This is illustrated in Fig. 6 where the PCA data for conventional (Fig. 6a) and capillary HPLC-MS data are shown (Fig. 6b). As this figure shows, with the conventional HPLC-MS system there is a trend, but no clear separation of the a.m. and p.m. samples. In contrast, when such analysis is performed with the capillary there is a very clear separation. Examination of the

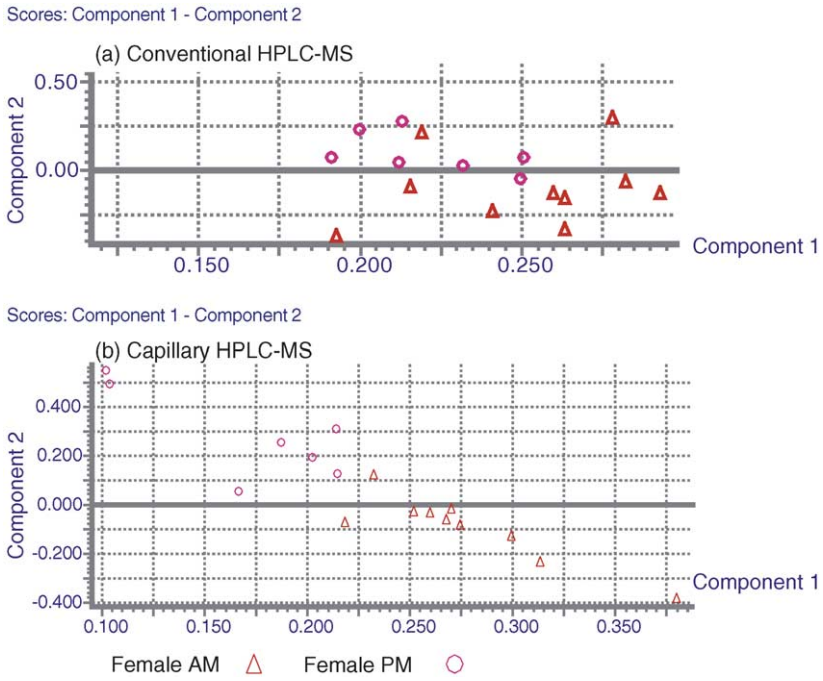

Fig. 6. Scores plots for PCA of both (a) conventional HPLC-MS and (b) capillary HPLC-MS data from analysis of female rat urine obtained from am and pm timepoints. As a result of the increased number of detected components in the capillary data, improved clustering based on diurnal variation is observed in the scores plots.

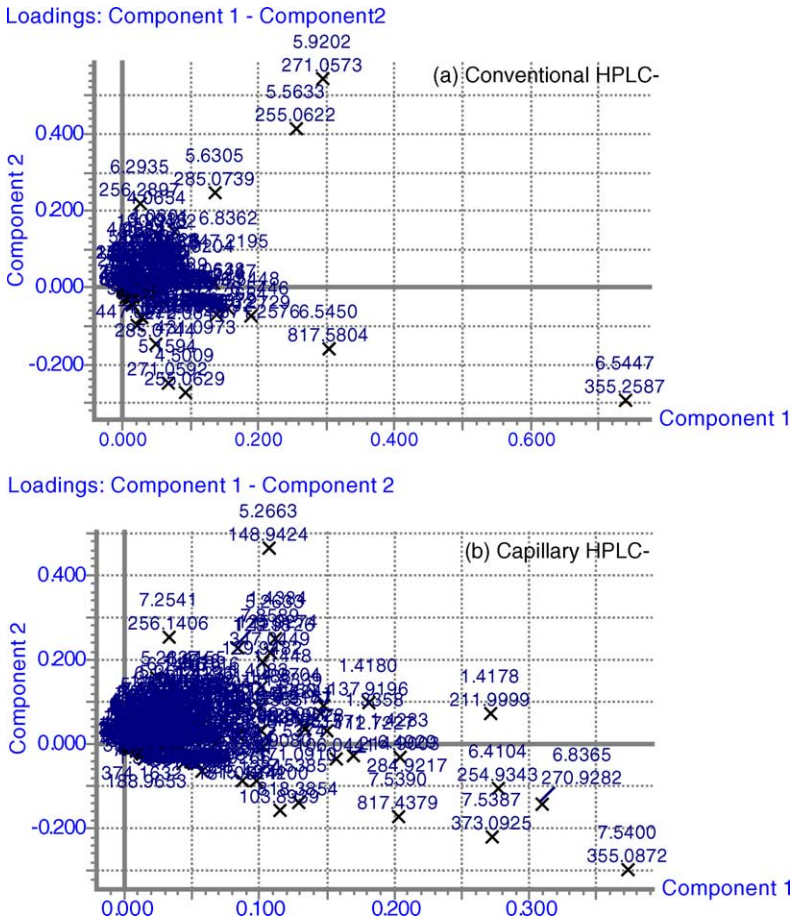

Fig. 7. Corresponding loadings plots for the scores plots presented in Fig. 6 for (a) conventional HPLC-MS and (b) capillary HPLC-MS. As expected, the loadings plot for the capillary HPLC-MS data contains more contributing ions than that for the HPLC-MS data.

loadings plots shown in Fig. 7a and b clearly shows that many more ions are contributing to the separation for the capillary results (Fig. 5) compared to the corresponding conventional HPLC-MS results (Fig. 3).

5. Multi-dimensional chromatography versus multi-column separations

A further problem associated with complex biofluid samples, such as urine or plasma, is that they contain a very diverse range of compound classes from polar neutrals such as sugars, which are barely retained on C18 bonded materials, through polar acidic and basic compounds, amphoteric substances and less polar materials of a similar nature. Retaining and eluting all of these diverse metabolites using single column chemistry is probably not feasible. However, to obtain as comprehensive a metabolite fingerprint as possible has to be a major aim in HPLC-MS-based metabonomics. This, therefore, leads inevitably to either multiple analysis of samples using different column technologies optimised for different compound classes or to the use of coupled column techniques combining, e.g., ion exchange and reversed-phase column chemistries to provide a degree of multidimensionality to the separation. An example of the type of separation that can be obtained using a prototype polymeric MCX column, tailored to provide a better separation of polar compounds, rather than a C18 bonded phase is given in Fig. 8. Similarly, microbore hydrophilic interaction chromatography (HILIC), using polyhydroxyethyl A and TSK Gel Amide 80 columns, combined with MS has been used for metabolomic analysis of polar plant metabolites [10]. Whilst we have not yet fully investigated the use of such systems in detail the eventual application of both mutli-column analysis and coupled-column chromatographies to metabonomic analysis seems logical and inevitable.

6. Developments in high resolution HPLC-MS-based metabonomics

Whilst capillary HPLC methods provide one means of obtaining an increase in the peaks detected in a sample by reducing the chemical noise entering the mass spectrometer, alternative strategies are becoming available as the result of the introduction of the combination HPLC systems using

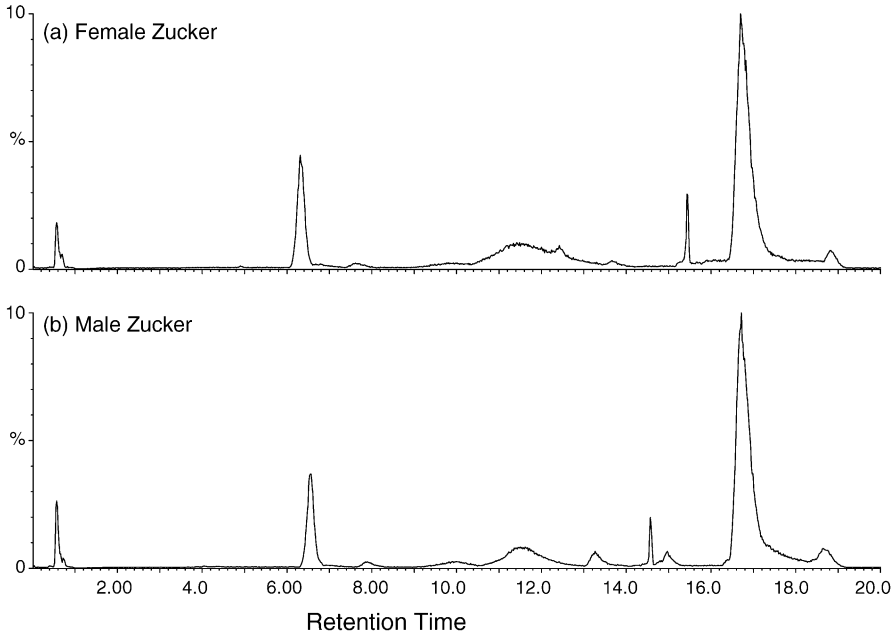

Fig. 8. Representative total ion current chromatograms for (a) female and (b) male Zucker rat urine analysed by HPLC-MS on a prototypical polymeric ion exchange column tailored to separate polar compounds.

sub 2 μm packings combined with high operating pressures. This technology ("UPLC") employs 1.7 μm porous particles, resulting in higher peak capacity, greater resolution and increased sensitivity compared to a 3 μm material. Indeed, the analysis of a Zucker rat urine sample by UPLC with a 1 min run time enabled a total of some 1000 peaks to be detected (data not shown). This approach, therefore, allows similar results to be achieved to those obtained previously by conventional HPLC, but in one tenth of the time. This radical shortening of the analysis time opens up the possibility of relatively high throughput screening for metabonomics. Alternatively a longer run (e.g., 5 min) can be employed and the number of peaks detected increased to ca. 5000 as shown in Fig. 9 (Plumb et al., in preparation).

7. The role of HPLC-MS in metabonomic investigations

The studies reported in the literature and described here clearly indicate that HPLC-MS has the potential to provide an immensely useful means of generating metabolite profiles. If hyphenated techniques are to become an important component of metabonomic studies, as seems very likely, then HPLC-MS may offer some advantages over the GC–MS techniques currently used in metabolomics. In particular, the ability to run samples with minimal sample preparation by HPLC-MS is in contrast to the more extensive sample pre-treatment techniques required to obtain comprehensive metabolite fingerprints by GC-based techniques (which is not to say that GC–MS will not find an important place in metabonomic investigations). For the potential of HPLC-MS to be realised, however, a number of problems and issues need to be addressed. One problem associated with the use of HPLC-MS-based metabonomic analysis at the moment is that the bulk of the thousands of ions detected are unidentified. As a result, whilst the rapid analysis of metabolite profiles obtained by these methods can be undertaken to generate "markers" it is not always as easy to determine what these molecules are. It is quite clear that a major effort is needed to identify and database as many of these endogenous metabolites (i.e., to map the metabonome) as soon as possible if the contribution of HPLC-MS in metabonomics is to be maximised.

Considerable care also needs to be taken in interpreting the data obtained from metabonomic studies where animals have been dosed with drugs or other toxins to ensure that the "markers" detected are not merely the excipients (e.g., PEG, cyclodextrins, DMSO, etc.) used to prepare the dose formulations. Similarly, as discussed earlier, great care must also be taken to prevent drug metabolites from being identified as "markers" associated with toxicity, pharmacology or disease modification, etc. To an extent, similar concerns may surround, e.g., dietary components. Ions for the latter may well feature in the loadings plots following a toxic insult or drug administration and whilst a decrease in excretion of a food-derived component in urine may indeed reflect toxicity such as, e.g., kidney damage it may equally reflect inappetence leading to reduced food consumption.

As we have noted above, HPLC-MS and NMR seem to provide valuable complementary information and analysis of samples, by both methods promises to provide an analytical strategy of great power. We can therefore envisage an analytical strategy whereby, in initial studies, sample fingerprinting

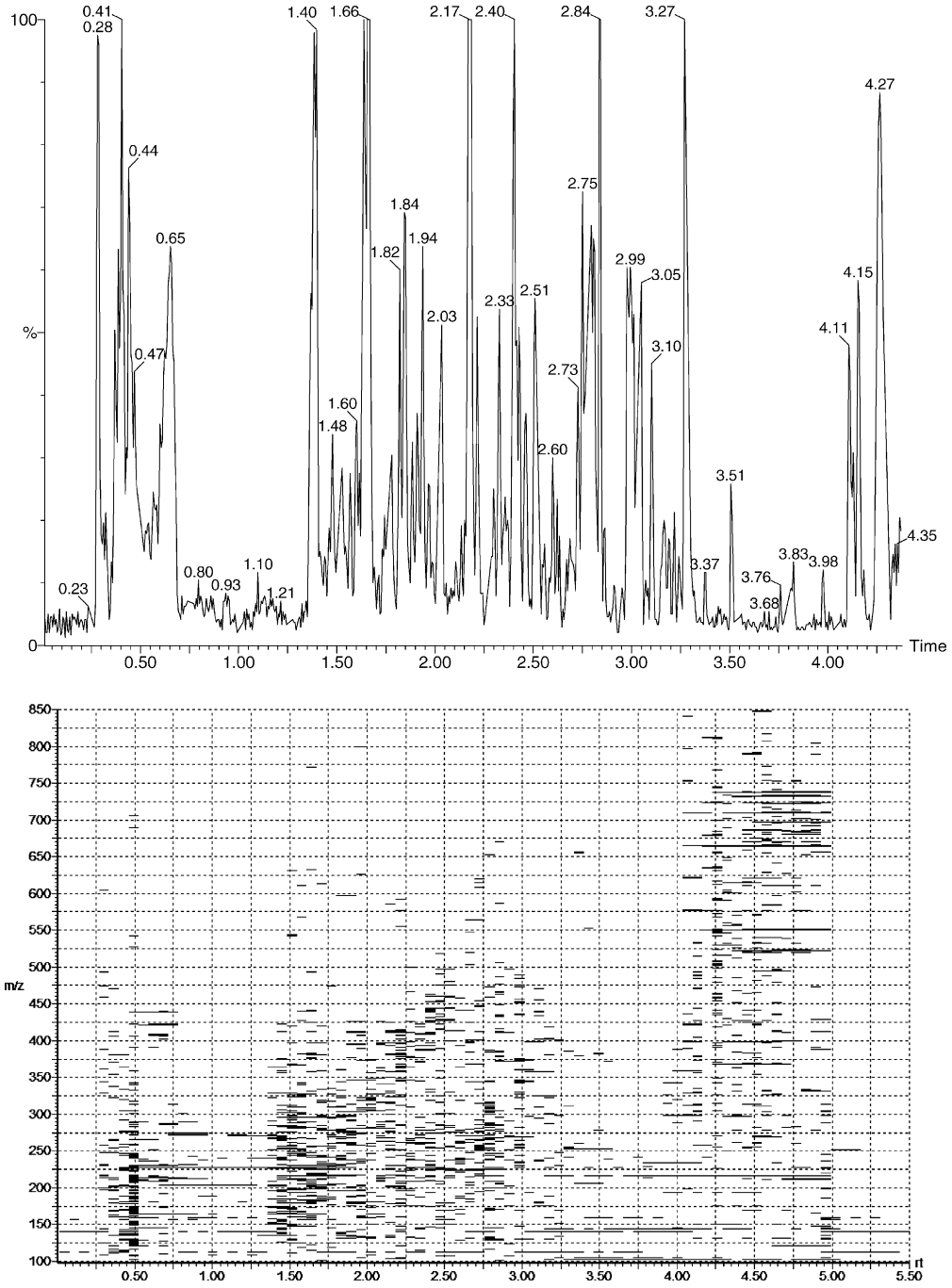

Fig. 9. A UPLC-MS separation of rat urine on a 3 cm × 2 mm 1.7 μm C18 bonded column using a 0–95% acetonitrile gradient over 5 min at 600 μl min and ca. 12,000 psi. Upper trace, total ion current, lower 2D mass chromatogram.

might be performed using both HPLC-MS and NMR techniques. Following the detection and identification of suitable markers for the system under study further work might then be performed using only HPLC-MS, with perhaps some modification of the analytical methods employed to also include the NMR-detected markers if appropriate. Given the relatively widespread availability of HPLC-MS systems compared to high field NMR spectrometers such a strategy has advantages in terms of efficient use of the available analytical resources.

8. Future prospects

The development of reliable HPLC-MS systems has been of great benefit in areas such as drug bioanalysis. The application of this technology to metabolite fingerprinting for metabonomic analysis was inevitable given the great potential of this type of technology. Our initial studies, and those reported in the literature to date and reviewed above, do indeed demonstrate considerable promise for HPLC-

MS-based analytical strategies for metabonomic research. Further the indications are that the use of capillary and very high pressure HPLC methods will improve the coverage of the metabonome provided by HPLC-MS as a result of reduced ion suppression. The widespread availability of high-resolution HPLC-MS/MS systems will inevitably result in a great increase in the use of this type of analytical approach for obtaining metabolite fingerprints. This will be particularly the case as an appreciation of both the potential for metabonomics to answer important biological questions becomes more widely known and the relative cheapness of the technology compared to, e.g., genomics and proteomics comes to be appreciated.

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