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Ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry

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Ultra-performance liquid chromatography (UPLC) utilizes sub-2 μm particles with high linear solvent velocities to effect dramatic increases in resolution, sensitivity and speed of analysis. The reduction in particle size to below 2 μm requires instrumentation that can operate at pressures in the 6000–15 000 psi range. The typical peak widths generated by the UPLC system are in the order of 1–2 s for a 10-min separation. In the present work this technology has been applied to the study of *in vivo* drug metabolism, in particular the analysis of drug metabolites in bile. The reduction in peak width significantly increases analytical sensitivity by three- to five-fold, and the reduction in peak width, and concomitant increase in peak capacity, significantly reduces spectral overlap resulting in superior spectral quality in both MS and MS/MS modes. The application of UPLC/MS resulted in the detection of additional drug metabolites, superior separation and improved spectral quality. Copyright © 2004 John Wiley & Sons, Ltd.

The detection and identification of drug metabolites is crucial to both the drug discovery and development processes, although in these two areas the emphasis is slightly different. In discovery, where the number and diversity of compounds is high, the focus is on detecting and identifying the major metabolites in a rapid manner. The data generated here will give information on rate and route of metabolism, as well as information on the cytochrome p450 isoforms involved in, or affected by, the metabolism of the compound under test.[1]

In contrast, in drug development, the number of compounds is less but the focus is on the detection and characterization of all of the metabolites. This exhaustive characterization of the metabolites is essential from a drug safety and patent protection perspective. As part of the regulatory submission it is necessary to prove that metabolites detected in man have also been observed during the toxicology studies with sufficient exposure margin to ensure safety. If a new compound is detected during human studies it will be necessary to either prove exposure in a new toxicological species (possibly primate) or to synthesize the compound and conduct a new safety assessment study on this new compound, as the metabolite itself may be toxic. From a patent point of view, it is essential to identify all the metabolites and assess their efficacy, as one of these new metabolites may be either the active moiety or more active than the candidate pharmaceutical.

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Liquid chromatography/mass spectrometry (LC/MS) and LC/MS/MS have become the mainstay of the drug metabolism scientist[2] and, when used in combination with LC/NMR,[3] it is possible to fully characterize any new drug metabolite. The invention of the atmospheric pressure ionization interface in the late 1980s allowed the facile connection of LC to quadrupole and triple-quadruple mass spectrometers. In the following years, the capability and functionality of mass spectrometers has increased. The triple-quadrupole mass spectrometer allowed not only *m/z* information, but also fragment ion information to be generated, allowing experiments such as neutral loss and product ion spectra to be performed. The introduction of ion trap mass spectrometers allowed MS^n spectra to be produced, allowing more information to be generated on new drug metabolites to help confirm structures. This capability was exploited, e.g., by Dear *et al.*,[4] who employed MS^3 and MS^4 to help characterize *in vivo* drug metabolite structures. The development of the hybrid quadrupole-othogonal time-of-flight mass spectrometer allowed the generation of exact mass information with greater accuracy and precision; these mass values can then be used to produce candidate empirical formulae which, at the 3–5 ppm error range, significantly reduce the number of possible structures of putative metabolites with molecular masses of a few hundred Da. This technology has also been used by Eckers *et al*. to identify trace impurities in drug formulations.[5]

The key to successful metabolite identification is detection and spectroscopic analysis. The detection of the drug metabolites relies upon good chromatography and a sensitive,

specific detector. In the drug development arena, radiolabeled material is normally available and thus LC/MS can be used in combination with liquid scintillation counters or radioflow detectors. The specificity of the radiochemical detector allows the drug metabolism scientist to focus only on the key chromatographic peaks of interest. Critical to this whole activity is the quality of the separation process. It is essential to effect complete resolution of the drug metabolites, ideally from each other and from all the endogenous components in the matrices. Failure to do so may result in the non-detection of low concentration metabolites or misassignment of structural isomers, e.g. glucuronides.

The LC separation of metabolites is usually performed in a gradient reversed phase mode, where the duration of the gradient, the particle size, the column length, and the linear velocity of the mobile phase control the resolution. The development of the chromatographic method is usually a compromise between desired resolution and analysis time. A careful review of chromatographic theory shows that a reduction in the stationary phase particle size has the greatest benefit to the chromatographic process.[6] Reducing the particle diameter from 5 to 1.7 μm will, in principle, result in a 3-fold increase in efficiency, a 1.7-fold increase in resolution, a 1.7-fold increase in sensitivity, and a 3-fold increase in speed. However, these benefits do come at a price; reducing the particle size by a factor of 3 results in an increase in the backpressure by a factor of 27, necessitating a new, purposely designed, chromatographic instrument to take advantage of such technology.

This paper describes the use of such a chromatographic instrument with columns packed with a 1.7 μm stationary phase for the analysis of drug metabolites of Midazolam in bile, possible the most challenging matrix we are faced with in drug metabolism. The results presented below demonstrate the speed, additional sensitivity, resolution and spectral clarity produced by this system.

EXPERIMENTAL

Chemicals

Acetonitrile (HPLC grade) was purchased from J. T. Baker (NJ, USA), ammonium formate and formic acid (spectroscopic grade) were purchased from Sigma-Aldrich (MO, USA). Distilled water was purified 'in-house' using a MilliQ system (Millipore, MA, USA). Leucine-enkephalin was obtained from Sigma-Aldrich. Midazolam was purchased from Sigma-Aldrich (Poole, UK).

Liquid chromatography

HPLC was performed using a Waters® Alliance HT® liquid chromatograph (Waters Corp., MA, USA). Conventional separations were performed on a 100 × 2.1 mm Waters Symmetry® C_{18} 3.5 μm column, eluted with a linear gradient from 0.1% aqueous formic acid to 95% acetonitrile over 30 min at 400 μL/min. UPLC was performed using a Waters ACQUITY UPLC™ system, equipped with a binary solvent delivery system, an autosampler, and a tunable UV detector. The chromatography was performed on either a 100 × 2.1 mm Waters ACQUITY C_{18} 1.7 μm column or on a 100 × 1 mm Waters ACQUITY C_{18} 1.7 μm column. The 2.1 mm i.d. column was eluted with the same aqueous formic acid/acetonitrile gradient as above, over either 30 min at 400 μL/min or 6 min at 500 μL/min. The 1 mm i.d. column was eluted with the same gradient conditions at 100 μL/min.

Mass spectrometry

Mass spectrometry was performed using a Micromass Q-Tof-micro™ (Waters, Manchester, UK) operating in positive ion electrospray mode. The nebulization gas was set to 500 L/h at a temperature of 250°C, the cone gas set to 0 L/h and the source temperature set to 110°C. The capillary and cone voltages were set to 3000 and 35 V, respectively. The Q-Tof-micro instrument was operated in wide-pass quadrupole mode for MS experiments; the TOF data were collected between *m/z* 50 and 850 with a low collision energy of 10 eV. The acquisition rate was set to 0.3 s/spectrum. The MS/MS experiments were performed using a collision energy of 25 eV. All analyses were acquired using an independent reference lock-mass ion via the LockSpray™ interface to ensure accuracy and reproducibility. Leucine-enkaphalin was used as the reference compound (*m/z* 556.2771) at a concentration of 50 fmol/μL and flow rate of 30 μL/min. Data were collected in centroid mode with the LockSpray frequency set at 5 s and averaged over 10 spectra.

Animal studies

Rat bile was obtained from a bile duct cannulation study in which Midazolam was given at 5 mg kg^{-1} as part of the anaesthesia regimen. Samples were taken 1 h after dosing, frozen at −20°C, and then diluted 1:10 with 0.1% formic acid prior to analysis.

RESULTS AND DISCUSSION

The major benefit expected from the use of the sub-2 μm particles is the increased column efficiency and concomitant increased resolution. Figure 1(a) shows the base peak intensity (BPI) chromatogram for a 5-μL injection of diluted rat bile onto the HPLC system. The results obtained can be compared with the data produced for exactly the same sample and injection volume on the UPLC system (Fig. 1(b)); here it is apparent that the resolution is dramatically improved, with the number of discrete peaks more than doubled, illustrating the resolving power of the UPLC technique. The peak widths generated were of the order of 6 s at the base, giving a separation peak capacity of the order of 300, more than twice that produced by the HPLC system (Fig. 1(a)). The UPLC separations were performed at an initial column backpressure of 7000 psi (438 bar).

The metabolism of Midazolam is relatively simple; two primary sites of oxidation and glucuronidation have been previously described.[7] Two additional compound-related chromatographic peaks were observed in the bile samples from the present study. The positive ion electrospray mass spectra from these peaks contain MH^+ ions with a nominal *m/z* of 548. This value could correspond to a possible metabolite of Midazolam with double hydroxylation, O-methylation, and conjugation with glucuronic acid. The

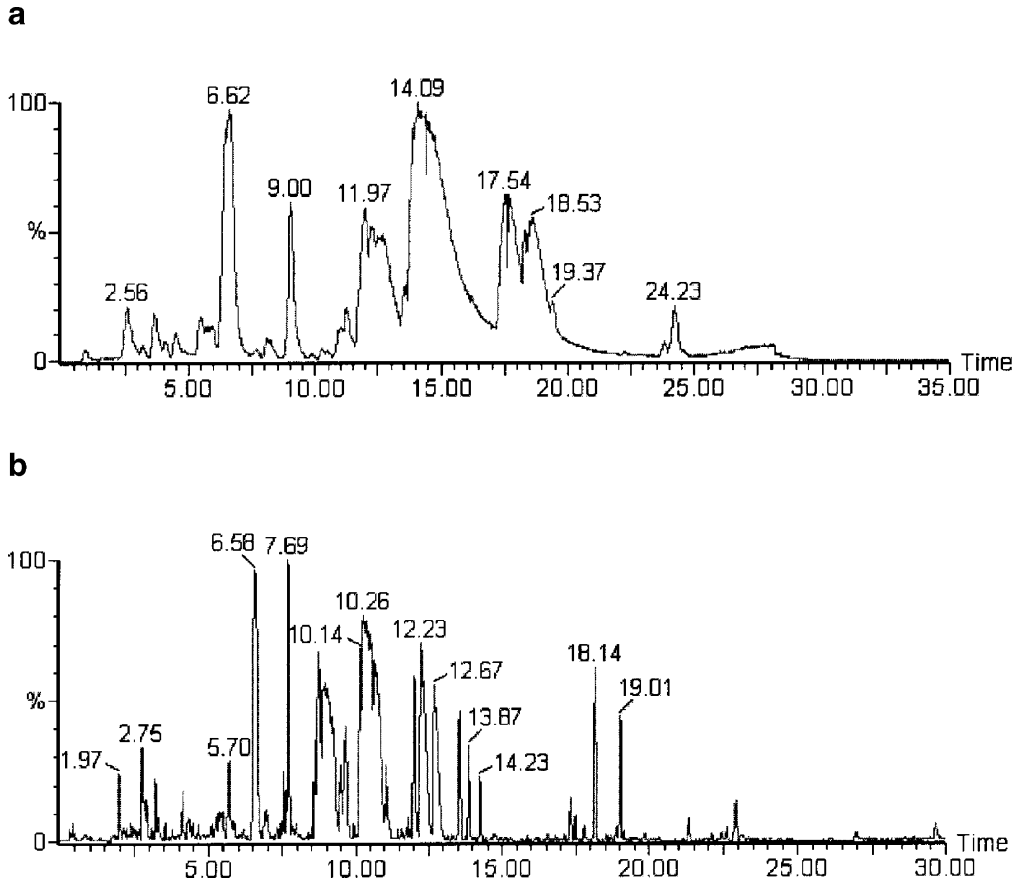

Figure 1. Separation of rat bile following the administration of Midazolam at 5 mg kg^{-1}: (a) 30 min separation on a 2.1 × 100 mm 3.5 μm C_{18} HPLC column and (b) 30 min separation on a 2.1 × 100 mm 1.7 μm C_{18} UPLC column.

hydroxylations can reasonably occur at the α-carbon atom and at the 4-position of the molecule (Fig. 3). For the two peaks observed, if this tentative assignment is correct, one of the hydroxyls is methylated and the other is glucuronidated. The extracted ion chromatogram for the $[M+H]^+$ ion at *m/z* 548 from the HPLC analysis is given in Fig. 2(a), and that obtained from UPLC in Fig. 2(b). From the data obtained from these two injections it is clear that in the UPLC system the two glucuronides are clearly resolved (labeled 1 and 2) from each other, while they were not resolved by the conventional HPLC system. This extra resolution has a beneficial effect upon the resulting spectra; the data in Fig. 4(a) shows the glucuronide peak in the HPLC analysis, and Fig. 4(b) shows the extracted spectra of the glucuronide peak 1 from the UPLC analysis. As we can see from this data, for the UPLC-derived spectrum, *m/z* 548 is the dominant ion in the spectrum whilst, in the HPLC analysis, *m/z* 548 is a minor contributor thus making data analysis significantly easier with the UPLC data. The measured exact mass value of 548.1248 gives a mass error of 1.8 ppm.

This data illustrates the benefit obtained from the extra resolution of UPLC both in terms of specificity and spectral clarity. One concern when operating with these very narrow peaks is the ability of the MS instrumentation to obtain a sufficient number of data points across the peak to perform peak integration and data-dependent MS/MS analysis. The faster duty cycle of the TOF instrumentation should allow rapid data collection and good quality MS/MS analysis. To test this hypothesis we performed a data-dependent neutral loss experiment on the Midazolam bile sample to search for glucuronidated conjugations. The neutral loss experiment (*m/z* 176.0321) was performed using alternate high/low collision energy; upon detection of this loss the instrument switches to MS/MS analysis of the precursor ion, the data obtained is shown in Fig. 5. The Q-Tof instrument was switching with a frequency of 300 ms and we can see that this frequency was sufficiently rapid to maintain the chromatographic information while still obtaining good MS/MS data. The MS/MS data produced many acquisitions for glucuronide conjugations, which were present in the sample. However, in particular, we can concentrate on the previous glucuronide at *m/z* 548 mentioned before. The main fragment ion gave a mass measurement value of 372.0904, which, for $C_{19}H_{16}N_3O_2FCl$, resulted in mass accuracy of 3 ppm. A further example of the separating power of UPLC is the resolution of the further hydroxylated glucuronide Midazolam metabolites with *m/z* 564. The data produced by the UPLC and HPLC systems is shown in Fig. 6 and again we can see that the UPLC system clearly resolves the two metabolites that are unresolved in HPLC. In this paper we have chosen to use a time-of-flight mass spectrometer due to the high data acquisition rates and to ensure that we have sufficient data points across the LC peaks. Subsequent work (data not shown) with a quadrupole mass spectrometer has shown that it is necessary to have a data capture rate of 10–20 scans per second in order to obtain high-quality data. Thus it should be

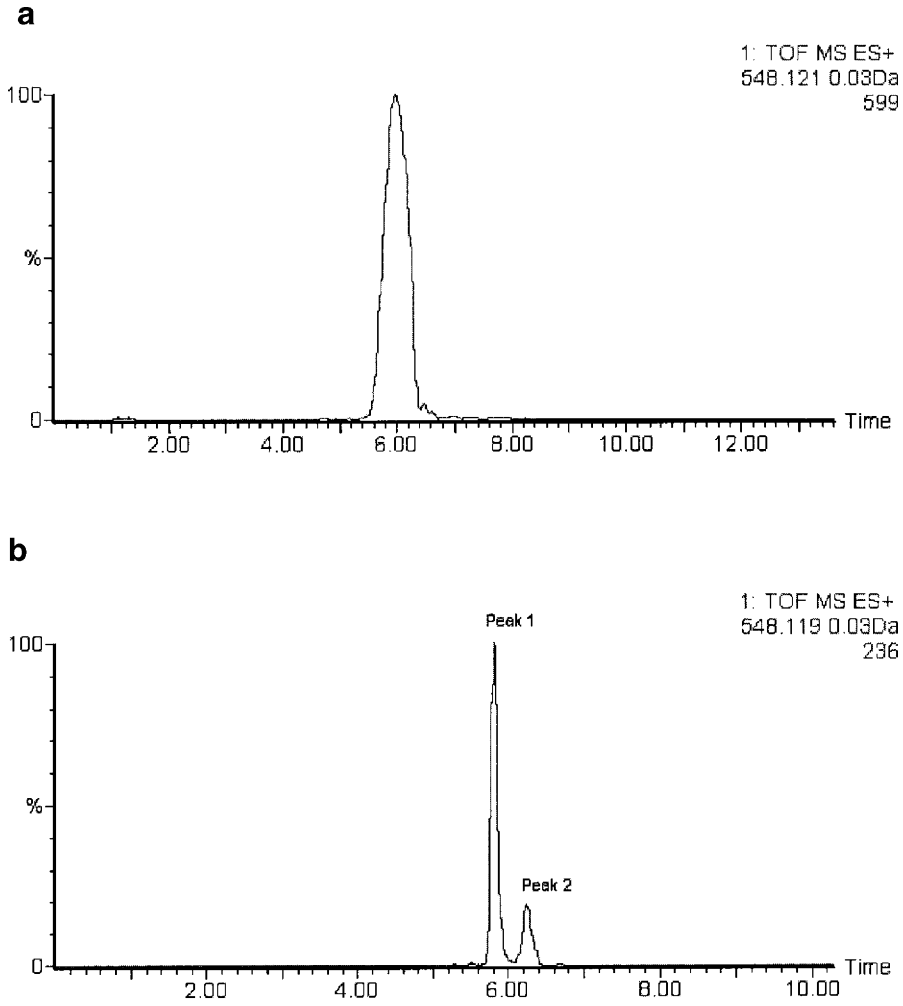

Figure 2. Extracted ion chromatograms at *m/z* 548 for (a) HPLC and (b) UPLC.

possible to obtain high-quality UPLC data with modern quadrupole and ion trap mass spectrometers.

In the field of drug discovery there is a desire to increase throughput to cope with the increased number of compounds being submitted for metabolic analysis and to facilitate faster decision-making. In order to address this requirement we took advantage of the flatter nature of the optimal operating linear velocity in the van Deemter plot for sub-2 μm materials and operated the system at higher flow rates. The UPLC separation was performed with a gradient time of 6.5 min using a flow rate of 500 μL/min, resulting in a backpressure in the order of 9000 psi. Again if we extract the *m/z* 548 ion we can see that the two peaks are still clearly resolved (Fig. 7). This data illustrates that with an approximate 5-fold reduction in analysis time, UPLC offers superior resolution to that produced by HPLC. The real advantage of UPLC is producing superior quality data in a shorter period of time. An alternative approach to obtaining high-throughput and high-resolution separations would be to use a monolithic column, as shown by Dear *et al.*[8] However, in order to generate separations with the same resolving power in the same time as that shown with the UPLC instrumentations would result in monolithic column lengths of approximately 50 cm and flow rates of 6–10 mL/min. This would result in a 20-fold dilution in the eluting peak concentration and also would mean that the column eluent would need to be split prior to introduction into the mass spectrometer. As with any new technology there are some initial issues, namely the need to purchase new instrumentation and also a limited range of columns. However, the authors feel that benefits in terms of increased sensitivity and throughput makes the investment worthwhile. The range of stationary phases, while limited at the current point in time, is sure to increase as interest in this new field of chromatography increases.

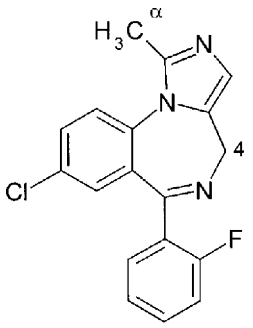

Figure 3. Structure of Midazolam.

The low dispersion volume of the UPLC system facilitates the use of 1 mm i.d. columns, which should provide

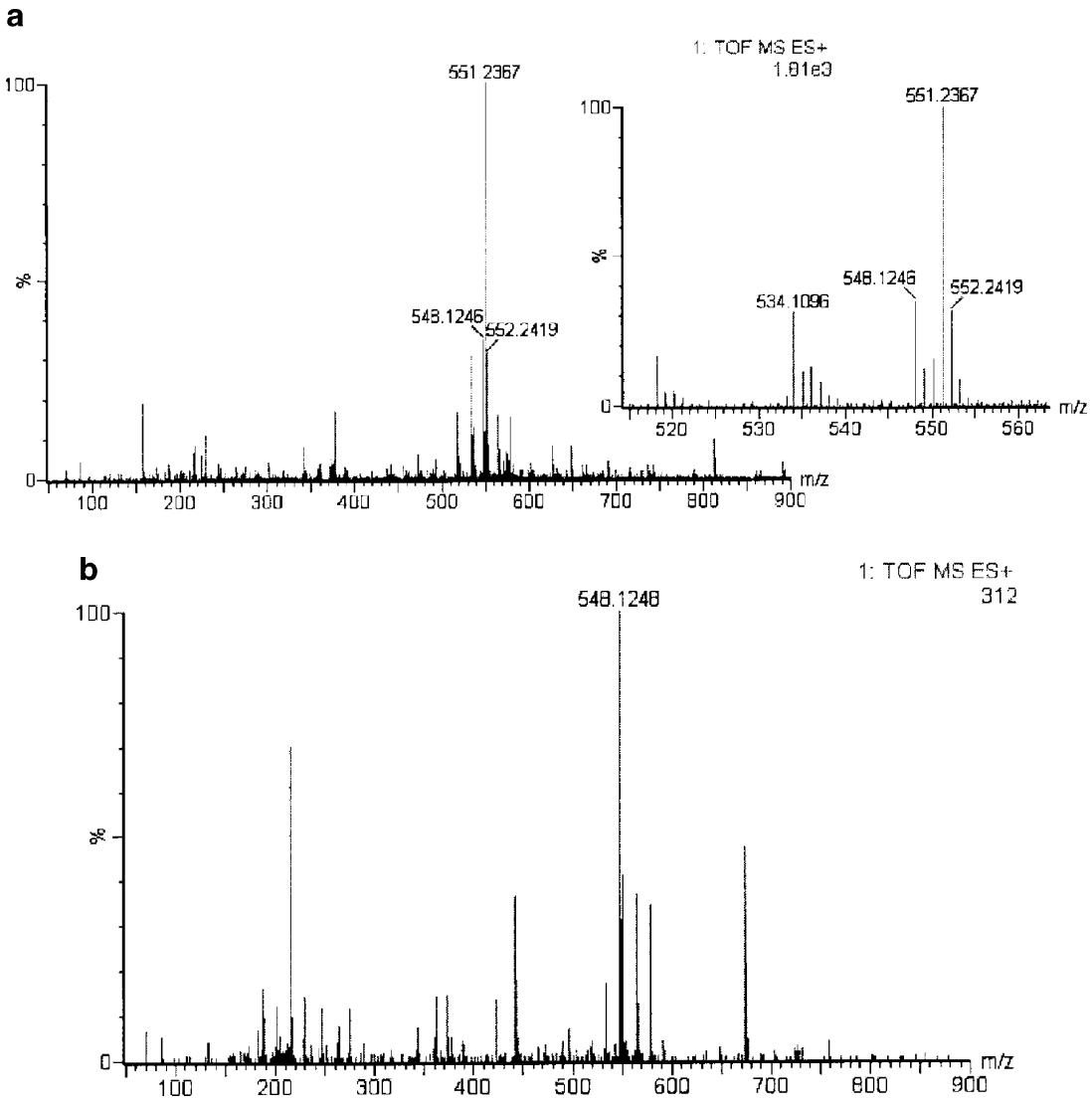

Figure 4. (a) Positive ion MS spectra of the HPLC glucuronide metabolite and (b) the spectra from the UPLC analysis of the glucuronide.

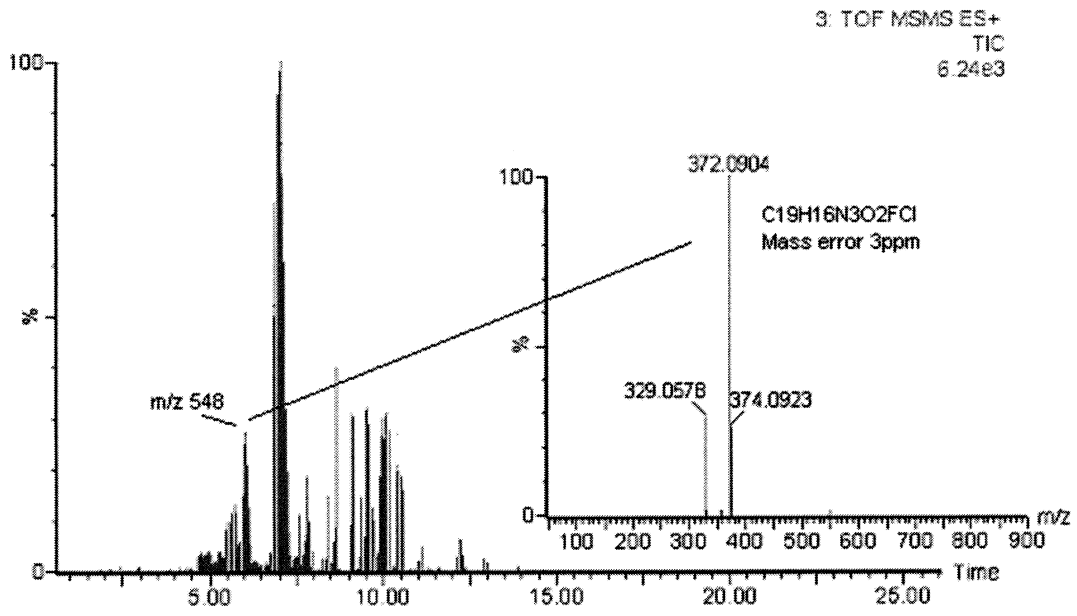

Figure 5. Neutral loss analysis of *m/z* 176.0321 for UPLC separation of rat bile. Inset is the extracted spectra of one of the *m/z* 548 Midazolam glucuronides.

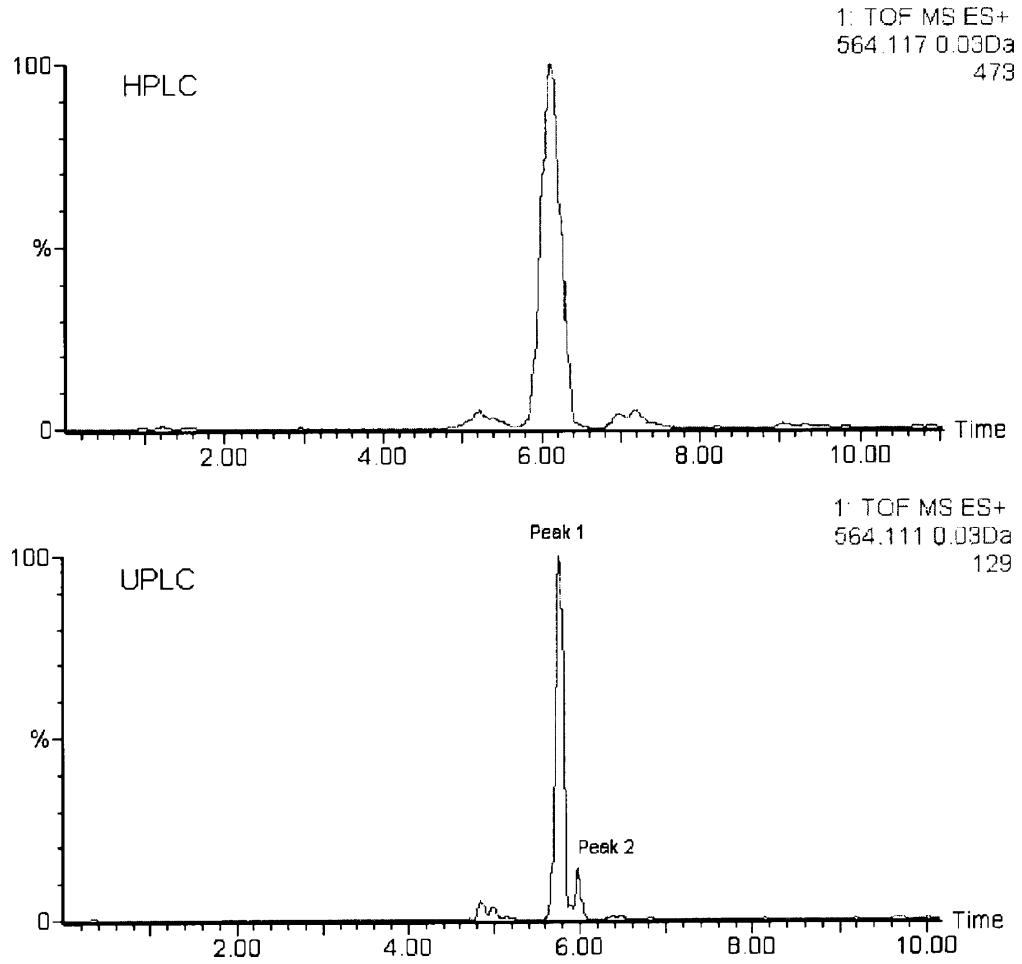

Figure 6. Extracted ion chromatograms for *m/z* 564 from HPLC (top) and UPLC (bottom).

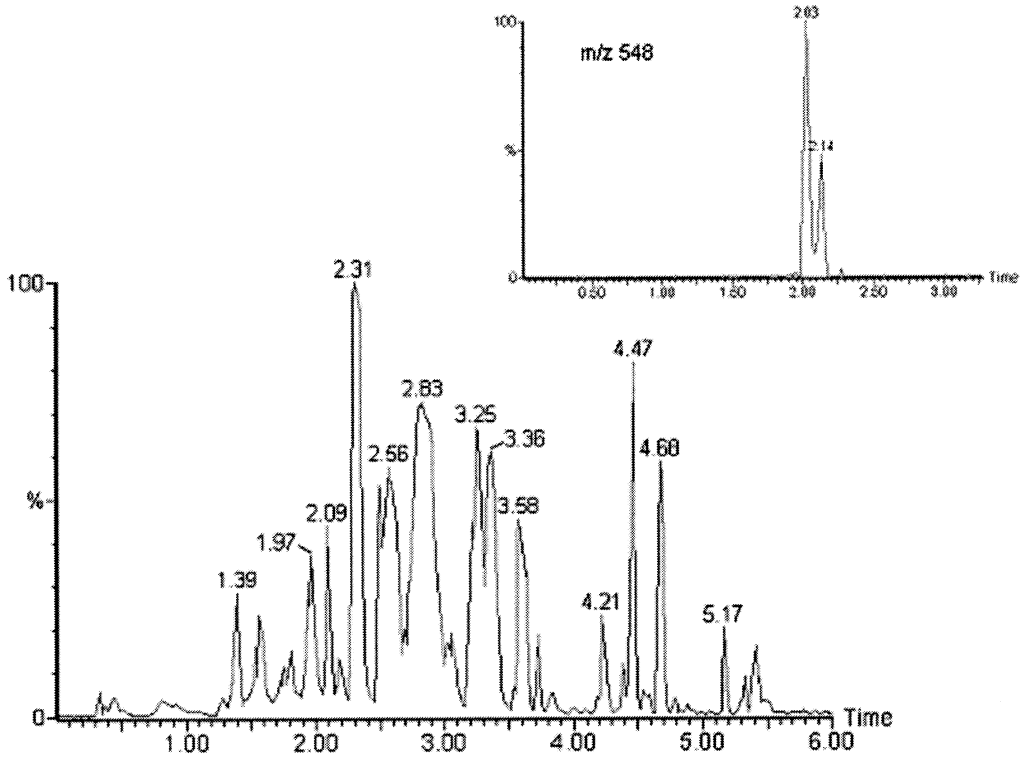

Figure 7. UPLC TIC separation of rat bile following the oral administration of Midazolam using a 2.1 mm × 10 cm 1.7 μm C_{18} UPLC column. The separation was performed with an analysis time of 6.5 min. Insert is the extracted ion chromatogram for *m/z* 548.

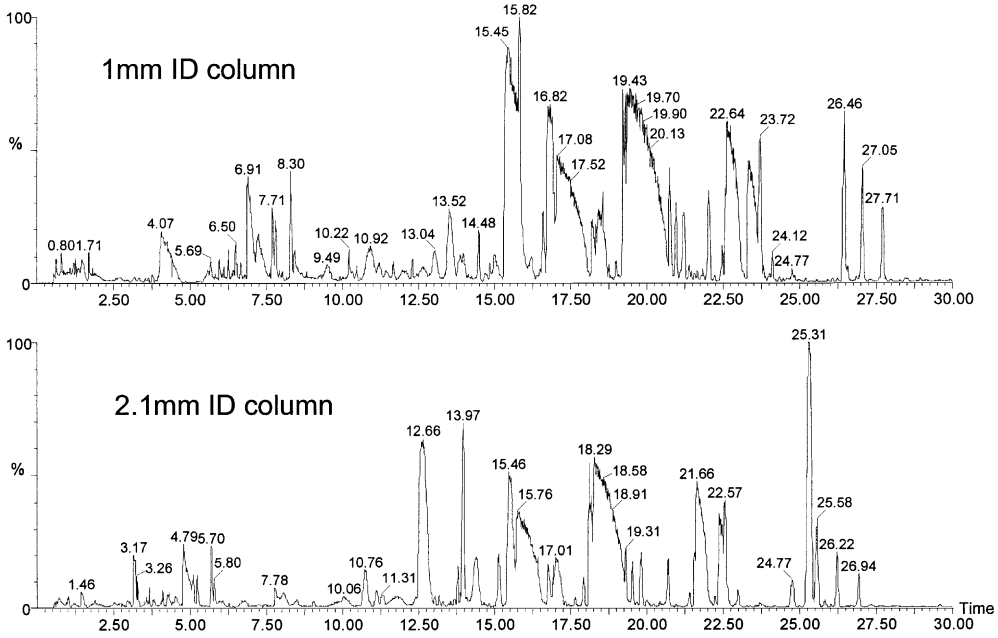

Figure 8. Comparative TIC traces obtained from the UPLC analysis of rat bile following the administration of midazolam at 5 mg kg^{-1}. Data from the 1 × 100 mm 1.7 μm C_{18} column is shown on the top and that from the 2.1 × 100 mm 1.7 μm C_{18} column is shown on the bottom.

increased sensitivity. However, up to this point in time, the use of 1 mm columns has not been popular as method transfer from 2.1 mm or 4.6 mm columns has proved difficult, especially for rapid gradient separations. The data in Fig. 8 shows the separation of the Midazolam rat bile on 2.1 and 1 mm columns. Both columns were eluted with a linear gradient of 0–50% aqueous formic acid/acetonitrile; 5 μL of sample were injected onto the 2.1 mm i.d. column and 1 μL of sample was injected on the 1 mm i.d. column. When the column geometry is reduced from 2.1 to 1 mm we should see a 4-fold increase in peak height; as we have injected 80% less onto the 1 mm column, the resulting peaks should be of similar intensity. In the data shown in Fig. 8 the two chromatograms have been normalized for response; as we can see the two chromatograms are of similar intensity, which agrees with the theory. The elution times of the peaks are slightly delayed on the 1 mm column compared to that of the 2 mm columns. This data shows that UPLC can be operated at the 1 mm scale with similar performance and throughput as 2.1 mm i.d. columns. One important factor in any chromatographic analysis is the reproducibility of the data; during the course of these studies we have determined that the reproducibility of this UPLC method is at least as good as conventional HPLC.

CONCLUSIONS

UPLC/MS produces a significant increase in peak resolution and spectral quality compared to HPLC/MS. The extra resolution provided by the UPLC system reveals new information about the samples under test and reduces the risk of non-detection of potentially important metabolites. The extra speed provided by the UPLC system allows the analytical run time to be reduced by factor of 5, whilst still maintaining the chromatographic resolution. In order to address the very narrow peaks produced by UPLC it is necessary to use a high data capture rate mass spectrometer such as a TOF or quadrupole with fast scan rates. We have shown that it is possible, using the Q-Tof micro, to generate MS and MS/MS data on these narrow peaks typically as low as 1.8 s wide.

UPLC has set a new standard for LC/MS in the analysis of complex mixtures in biological matrices. This raises the question that without using this technology are we detecting all of the analytes in our sample or separating the metabolic isomers. In the opinion of the authors this technology is one of the most significant advances in LC/MS since the electrospray interface. With this new approach we can gain more knowledge of samples we analyze in a faster time scale and thus make more informed decisions. If it is indeed true as Isaac Newton said ''If I have seen further it is by standing on the shoulders of giants.'' then with UPLC we see further information in our samples by realizing the chromatographic performance predicted by such greats as A. P. Martin, J. C. Giddings and J. H. Knox.

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