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Narrow-bore sample trapping and chromatography combined with quadrupole/time-of-flight mass spectrometry for ultra-sensitive identification of in vivo and in vitro metabolites

David J. Foltz [a], Jose Castro-Perez [b], Pamela Riley [a], John R. Entwisle [a], Timothy R. Baker [a,*]

[a] *Procter & Gamble Pharmaceuticals, Health Care Research Center, 8700 Mason-Montgomery Road, Mason, OH 45040-8006, USA*
[b] *Waters MS Technologies, Floats Road, Wythenshawe, Manchester M23 9LZ, UK*

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Abstract

The identification of in vitro and in vivo metabolites is vital to the discovery and development of new pharmaceutical therapies. Analytical strategies to identify metabolites at different stages of this process vary, but all involve the use of liquid chromatography separations combined with detection via mass spectrometry (HPLC/MS). Reported here is the use of narrow-bore column (0.5–1.0 mm i.d.) trapping of metabolites, followed by back-flushing onto a matching analytical column. Separated metabolites were then identified using quadrupole time-of-flight mass spectrometry (MS) and tandem MS. Metabolites in human plasma and from low-level in vitro incubations, that were not identified using standard HPLC/MS approaches, were characterized using the instrumental configuration described here.

Keywords: Mass spectrometry; HPLC/MS; Metabolite identification; Ultra-trace identification

1. Introduction

Mammals have a number of mechanisms for eliminating xenobiotics (foreign chemical substances). One of these is to metabolize or biotransform the chemical structure of a xenobiotic substance. Generally, metabolism is an enzymatic alteration (e.g., cytochrome P-450s) that produces a more polar chemical structure, thereby increasing a compound's hydrophilicity and facilitating renal clearance [1]. For a number of reasons this phenomenon is of interest to the research, development and finally administration of pharmaceutical therapies. Metabolism can begin, alter, prolong or, most often, halt a desired physiological effect. It can in rare cases produce a compound that has undesired toxicity, whereas the original drug did not. Additionally, efficacious metabolites must be patented to protect valuable discoveries.

In drug discovery, identification of metabolites generally takes place following incubations of new chemical entities (NCEs) in hepatocytes or sub-cellular fractions (e.g., S9 fractions). The purpose of these identifications is to locate metabolically susceptible sites, the specific locations on NCEs that are structurally altered. Feedback of this information contributes to the structure-activity relationship data that drives the medicinal chemistry program. Ideally, for NCEs that are not metabolically stable, a next generation of active molecules can be synthesized that are able to impede metabolism at vulnerable structural sites, thereby resulting in candidates with improved pharmacokinetic parameters (e.g., longer half-life or improved bioavailability).

As a drug candidate progresses into the development stage, it is necessary to understand the nature of its metabolism in humans and in species used for safety testing prior to human exposure (typically rat and dog). The result is considerable effort to define the chemical structure of significant metabolites, particularly those present in plasma (circulating exposure to the body). Typically, a radio-isotope-labeled analog

* Corresponding author. Tel.: +1 513 622 1380; fax: +1 513 945 5238.
E-mail address: baker.tr@pg.com (T.R. Baker).

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of the drug candidate is co-administered in order to quantitatively track the absorption, distribution and elimination of the drug and metabolites and to facilitate the identification of biotransformation products.

The combination of liquid chromatography and mass spectrometry has been the key technology, for more than a decade, to identify the structures of metabolites [2,3]. Although a number of different strategies and experimental sequencing are utilized [4–7], the basic analytical approaches employed are similar for elucidation of metabolites derived from either in vitro or in vivo sources. Typically, minimal sample preparation (none, dilution or protein precipitation) is followed by reversed-phase chromatography using 4.6–2.0 mm internal diameter (i.d.) columns to separate the analytes. This chromatography is most often interfaced to mass spectrometry detection using some form of electrospray volatilization/ionization. The mass spectrometers used are generally quadrupole, ion trap and more recently time-of-flight instruments [8,9]. Many different experimental approaches to identify metabolites fit into that general description, however, the widespread use of this technology attests to the power of the HPLC/MS technique and its ability to meet these analytical challenges.

There are challenges with respect to metabolite identification that are not readily solved by the commonly utilized approach outlined above. Increasingly potent drug candidates are being advanced, resulting in lower levels of compounds administered and still lower levels of metabolites requiring identification. This trend is, in some cases, challenging current practices. In other cases, identification of metabolites at relatively low percentages of overall drug-related signal are required to insure cross-species coverage for toxicology purposes. Commonly, the most challenging metabolites to identify are those in human plasma, the very situation where obtaining this information is most important. This is because the doses administered to humans are lower than in the toxicology species and the presence of radio-isotope-labeled analogs, that serve as markers, are strictly limited (typically 100 μCi total radioactivity). Finally, in some cases the identification of metabolites is challenged by the detection of specific analytes. This can be the case for metabolites that have poor intrinsic response to the atmospheric pressure ionization process (volatilization/ionization) or more commonly when the compounds of interest are poorly retained. In that situation their detection can be hampered by the largely aqueous mobile phase, early in the chromatographic gradient, and the high levels of salts and polar biological matrix components that can suppress the analyte signal.

A number of strategies have been employed to overcome these and other metabolite identification challenges. Fraction collection followed by concentration and re-injection or infusion (no chromatography), have been successfully utilized. These have disadvantages of requiring, sometimes significant, additional time as well as introducing unwanted sample manipulation that can chemically degrade the analytes and concentrate matrix interferences. Altering chromatography to overcome some challenges is often possible, but this has involved additional method development time and careful correlation with the initial separation (i.e., the metabolite elution order observed in the first separation). Larger sample volumes can be analyzed, but for direct injection chromatography the presence of increasing amounts of matrix quickly puts this simple approach into the category of diminishing returns. We have even attempted a peak parking approach [10] with capillary column chromatography, but had limited success. In sum, these strategies for overcoming challenging metabolite identification problems have been successful in many cases, but they tend to be slow, situation-dependant solutions.

We have configured a system that combined three well-known analytical technologies (advantages) that has proven to be superior to the problem-solving approaches described above. The system utilized narrow-bore column trapping, followed by back-flushing and analytical separation on an identical reversed-phase narrow-bore column. The separation was followed by detection with time-of-flight (Tof) mass spectrometry and tandem mass spectrometry (quadrupole/Tof). This system showed significant advantages for both in vivo and in vitro identifications of metabolites.

2. Experimental

2.1. Materials and reagents

HPLC grade acetonitrile and methanol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid (Suprapur®) was purchased from EMD Chemicals, Gibbstown, NJ, USA. Water was obtained from an in lab Milli Q (Millipore Corp., Billerica, MA, USA) water filtration system. Compounds tested were proprietary NCEs.

2.2. Sample preparation

The human plasma sample was collected following a 525 mg peroral administration of the drug. The plasma sample was prepared by first adding 5:1 acetonitrile to precipitate proteins. The supernatant was taken to dryness under nitrogen and then reconstituted in 10% acetonitrile. In vitro incubations of NCEs (0.25, 1.0 or 25 μM concentrations) were performed in S9 hepatocyte fractions and quenched with 50% methanol. Samples were then injected without further manipulation.

2.3. HPLC

Columns were purchased from Phenomenex Inc., Torrance, CA, USA. All columns employed the Luna C8(2) stationary phase with various dimensions (4.6 mm × 250 mm, 5 μm; 2.0 mm × 150 mm, 3 μm; 1.0 mm × 150 mm, 3 μm and 0.5 mm × 150 mm, 3 μm).

HPLC conditions for narrow bore chromatography included the use of two mobile phases, A (95% water, 5% acetonitrile, 0.1% formic acid) and B (95% acetonitrile, 5% water, 0.1% formic acid). Injections of in vitro samples onto the trapping column occurred for 3 min at 100% A, with the final injection holding for 6 min to provide additional washing. The analytical column and initial back-flush were at 20% B. A 15 min gradient, ramping to 90% B, began immediately following the switch of flow to the separation column, and was then held for 3 min. The separation column was then returned to 20% B and the trapping column to 100% A.

Analysis of in vivo samples utilized the same mobile phases. The 4.6, 2.0 and 1.0 mm i.d. columns utilized flow rates of 1.0, 0.3 and 0.040 mL/min, respectively. Starting conditions were 95% A for 10 min, followed by a 40 min gradient to 95% B and a 2 min hold with an 8 min recycle. For the narrow bore (1 mm i.d. column) experiments, a single 49 μL injection of sample was trapped and washed with 100% A for 10 min.

The sensitivity versus flow experiment was performed at an optimized temperature for each flow rate but not more than 85% of the maximum allowed by the instrument's software. Nebulizing gas was also optimized for each flow. The electrospray capillary was set to 3250 V and the cone was held at 30 V. These experiments were performed by infusing 5 μL/min of a selected compound dissolved in 65% acetonitrile which was teed into a stream of that same organic composition, varying the bulk flow from 10 to 1195 μL/min. Each measurement was the result of 60 summed scans.

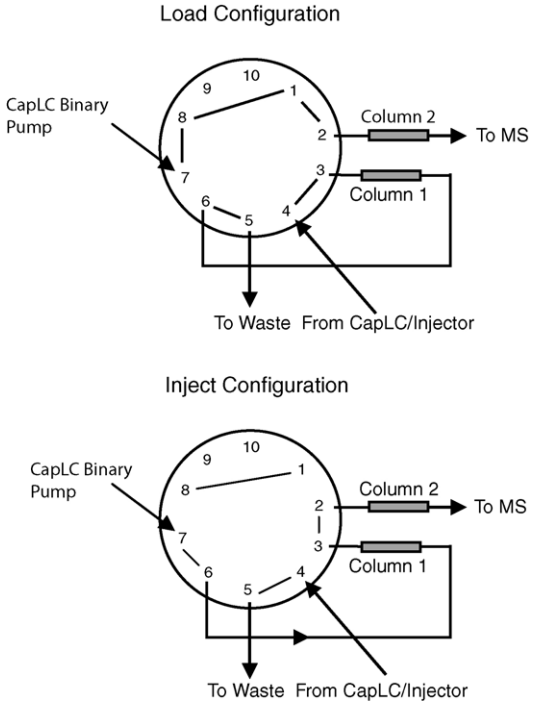

Fig. 1. Schematic diagram of column switching showing loading and washing of sample (top) and flow path for back-flushing and analytical separation (bottom).

2.4. Equipment

All HPLC/MS experiments were conducted on a Micromass Q-Tof API-US mass spectrometer (now Waters MS Technologies). Narrow-bore experiments used a Waters (Milford, MA, USA) CapLC (capillary liquid chromatograph). Experiments with 2.0 mm i.d. columns utilized a Waters Alliance HT 2795 HPLC system. The CapLC was fitted with a 100 μL syringe and a 2.4 μL sampling needle as well as a Micromass Stream Select Module (with 10-port valve), which was utilized to configure the columns and switch the flow path (Fig. 1). The Z-Spray mass spectrometer interface was fitted with a 60 μm i.d. stainless steel capillary and LockSpray® for internal calibration (using Leucine-Enkephalin).

To generate the HPLC/radioanalytical detection profile of the human plasma sample, HPLC fractions were collected using a Gilson FC204 (Middletown, WI, USA) configured with a Waters 2695 HPLC. Scintillation counting was done with a Packard Top Count NTX, using ASCII Flow II software.

3. Results

3.1. In vivo identification example

An example of the type of identification problem that was solved with the described experimental configuration is illustrated in Fig. 2. A mass balance study of a drug candidate was conducted in humans. The drug was co-administered to the subjects with 100 μCi of ^{14}C-labeled drug (as a radiotracer) and an equimolar amount of drug and $^{13}C_6$-drug. Because levels of the radiotracer in plasma were too low for on-line HPLC/radioactivity detection, fractions of the chromatographically separated sample were collected. Scintillation counting of the fractions allowed reconstruction of the radio-chromatogram shown in Fig. 2. The drug (base peak) and a number of metabolites are clearly seen in the reconstructed radio-chromatogram. With this profile (and matching chromatographic conditions) and the $^{13}C_6$-drug as a flag, the drug peak was confirmed and the identities of the majority of the metabolites were ascertained using HPLC/MS and HPLC/MS/MS with direct injection of protein-precipitated plasma onto a 2.0 mm i.d. column. The metabolites designated as M2 and M3, however, could not be identified,

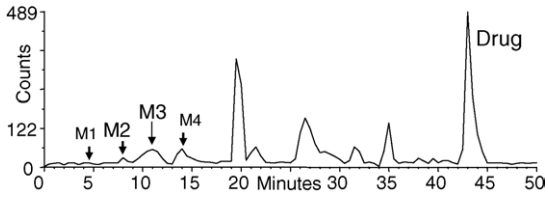

Fig. 2. Reconstructed radioactivity profile (scintillation counting of collected 1 min fractions) of drug and metabolites in human plasma. M2 and M3 could not be identified using conventional chromatography, fraction collection or other techniques.

even though the bracketing metabolites M1 and M4 were. A number of attempts to identify these two metabolites using approaches previously described (e.g., fraction collection, targeted MS/MS) failed to provide additional data. Although some indication of the potential structure of these metabolites was known from urine profiles, no definitive data could be obtained for these two metabolites within human plasma. Slight chromatographic differences with early eluting metabolites, across matrices, served to confound the issue. Identification of these two metabolites was desired to ensure a unique metabolite was not present in humans and that the toxicology species provided data inclusive of these two biotransformation products.

The two unknown metabolites (M2 and M3) were easily identified using trapping and elution with matching 1.0 mm i.d. columns. A retrospective interrogation of the original analysis (direct injection of 100 μL on a 2.0 mm i.d. column), compared with half as much sample analyzed with the narrow bore two-column arrangement, is provided in Fig. 3. Although some indication of M2 could be observed in the mass chromatograms from the direct injection on the 2.0 mm i.d. column, the mass spectrum (not shown) from these low signal/noise peaks was insufficient to provide an identification and MS/MS experiments on a signal of that magnitude would not have provided results. Clearly, the extracted ion chromatograms obtained with the narrow-bore arrangement indicate the presence of two metabolites and their SIL-analogs. The background-subtracted mass spectra, shown in Fig. 4 (left), were obtained from these chromatograms and they show the expected ratio of $[M+H]^+$ peaks for compounds that could be biotransformed from a 50:50 co-administration of a drug and $^{13}C_6$-drug analog (the amount of ^{14}C-drug was at trace levels relative to drug and SIL-drug and was not observed). Product ion spectra of the unlabeled $[M+H]^+$ ions, from a subsequent analysis of the two metabolites, are shown in Fig. 4 (right).

These results allowed a confident identification of metabolites M2 and M3. Importantly, these identifications proved that a unique metabolite was not present in man and both biotransformation products were also found in toxicology species. Even though the signals obtained were fairly low, the exact mass results obtained from the Tof mass spectrometer in the MS and MS/MS modes allowed close correlation to empirical formulae of the $[M+H]^+$ and fragment ions.

3.2. In vivo data considerations

In the example above, it is clear that the identifications could not have been made by direct injection of sample onto a 2.0 mm i.d. column. That initial analysis and the subsequent identification were performed with the same hybrid orthogonal quadrupole/Tof mass spectrometer. Although some benefit in concentrating the analyte was at least theoretically obtained in transitioning from an experiment using 2 mm i.d. columns to one using 1 mm i.d. columns, that concentration advantage, in this instance, was at least partially mitigated by the fact that half as much sample was introduced in the latter experiment. This was not by design, rather, it turned out that the required results were obtained with the first analysis of sample, a lower volume (of relatively precious sample) initially intended to test the system. For this particular example, it seems most likely that the key benefit was that

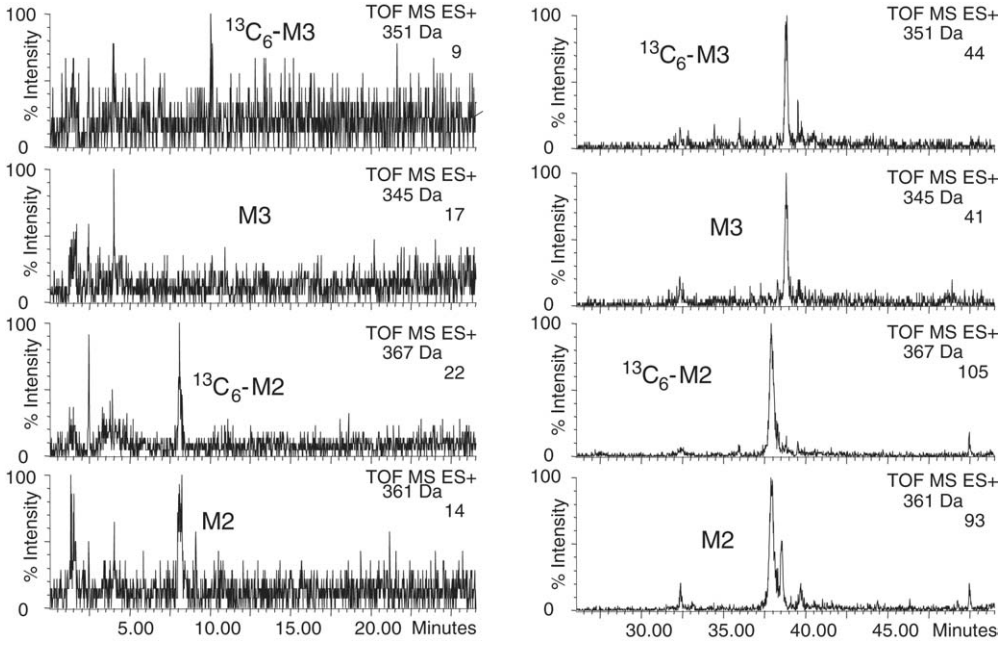

Fig. 3. Extracted ion chromatograms corresponding to the $[M+H]^+$ ions of M2 and M3 and their $^{13}C_6$-analogs following: direct injection of 100 μL of reconstituted human plasma extract onto a 2.0 × 150 mm column (on the left) and injection of 49 μL of the same sample with trapping, washing and elution using a pair of 1.0 × 150 mm columns (on the right).

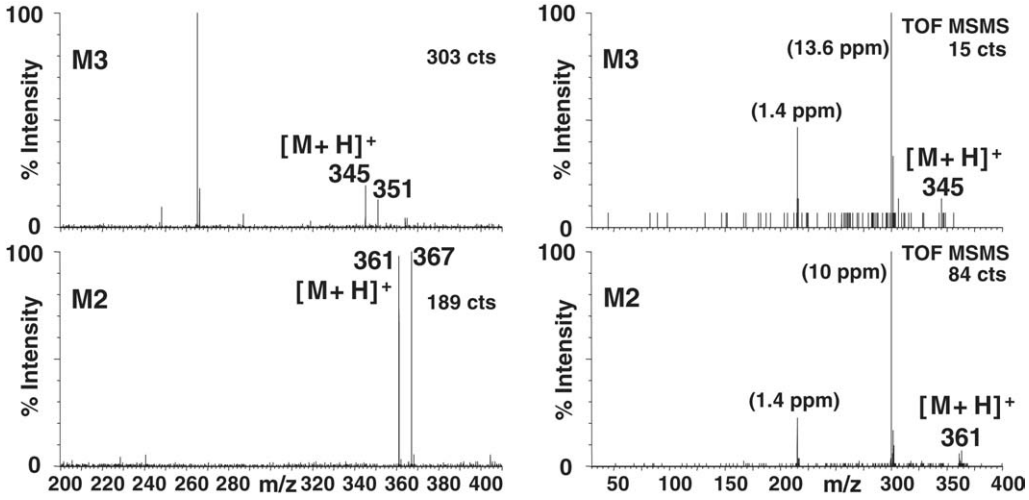

Fig. 4. Background subtracted mass spectra (left) of the two metabolites obtained from the narrow-bore analysis shown in Fig. 3. Product ion spectra from a subsequent analysis (on the right) showing fragment ions that allowed identification of the two metabolites. Experimental error for the accurate mass assignments of the fragment ions are shown parenthetically.

the sample was first loaded onto a trapping column and then washed with aqueous flow prior to back-flushing and elution onto the matching analytical column. The unknowns were early eluters and it is probable that poorly retained or trailing void volume matrix interferences were suppressing the electrospray ionization of the analytes of interest. Additionally, these relatively acidic analytes responded poorly, relative to other metabolites, in the electrospray source. Given the low level of the metabolites, however, the combination of narrow bore trapping and separation with sensitive accurate mass detection provided a confident identification. Although the use of identical stationary phases for both trapping and separation columns did not allow additional chromatographic selectivity, that choice was made to minimize the risk of altering the elution order of the metabolites since the identifications had to be correlated with the original quantitation via HPLC with radio-activity detection. The success with in vivo identifications encouraged further exploration with in vitro samples.

3.3. Metabolism from in vitro samples

NCEs have been incubated with metabolizing enzymes and then measured to determine loss of the starting material. This has allowed categorization or ranking of these NCEs. Measurement of NCEs in these incubations have been performed using quadrupole-based HPLC/MS or HPLC/MS/MS systems. These incubations were often carried out at relatively low concentrations (generally 1.0 μM) to more closely approximate therapeutic levels and avoid saturation of metabolic pathways. The sensitivity of MS-based determinations in the single ion monitoring (SIM) or selected reaction monitoring (SRM) modes readily allowed the use of these low level incubations. Often the observed metabolic susceptibility of an otherwise promising NCE instigated additional experiments to determine the identity of the metabolites formed. This typically required a second incubation of the NCE at a higher concentration (10–50 μM) in order to generate detectable levels of the metabolites of interest. With knowledge of the specific location of metabolic alterations, new NCEs were then synthesized with structural modifications designed to block or reduce those biotransformations.

3.4. Identifications from low-level in vitro incubations

Direct injection onto a 2 mm i.d. column, with mass spectrometric detection, has been our standard methodology for identification of metabolites from incubations with tens of μM concentrations of NCEs. An abundant signal was typically obtained from the full spectrum HPLC/MS acquisition of an NCE following a 25 μM incubation. Fig. 5 (top) illustrates an example of this. It shows a mass chromatogram of the $[M+H]^+$ ion, which exhibits a strong signal for this NCE. Metabolites of this NCE with lower abundance were also observed (not shown). Fig. 5 (middle and bottom) show that multiple injections trapped and then eluted onto 1.0 or 0.5 mm i.d. columns, with the same stationary phase, produced chromatographic peaks of similar intensity, from an incubation of 100-fold lower concentration of this NCE. The NCE appears to elute earlier in the direct injection example (Fig. 5, top), but the linear flow of mobile phase across the three different columns was similar. The delay reflects the time required for trapping and washing of the sample for the two other examples (Fig. 5, middle and bottom). Interestingly, all three chromatograms were obtained by injecting incubates that contained 50% organic solvent, as this was added to quench biological reactions. Retaining analytes from a 20 μL injection of this mixture onto a 2 mm column with a highly aqueous flow at hundreds of μL/min would be expected, however, retention from 20 μL injections onto 0.5 and 1.0 mm trapping columns at 15 and 40 μL/min flow rates, respectively, would not. In the latter cases it is possible that the analytes were not always trapped at the head of the

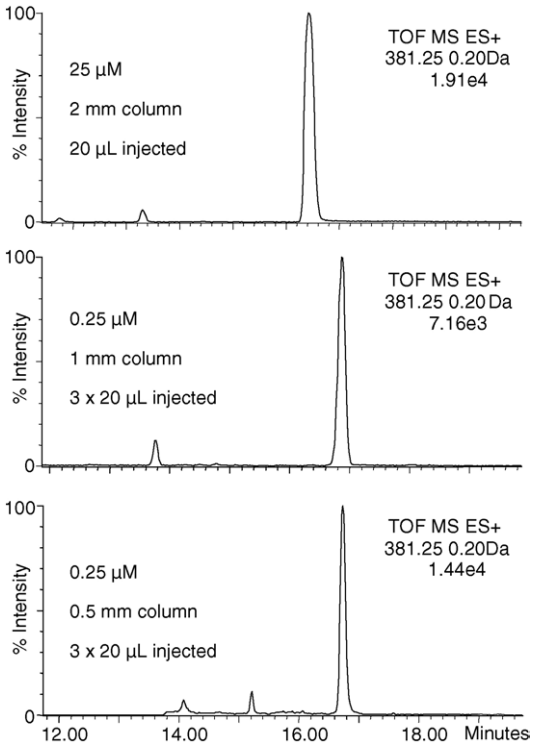

Fig. 5. Extracted ion chromatograms of the $[M+H]^+$ ion from a full spectrum LC/Tof-MS analysis of an NCE following incubations in rat S9 fractions. The top chromatogram was obtained from a direct injection, while the other two were obtained using trapping and then back flushing onto a matching separation column.

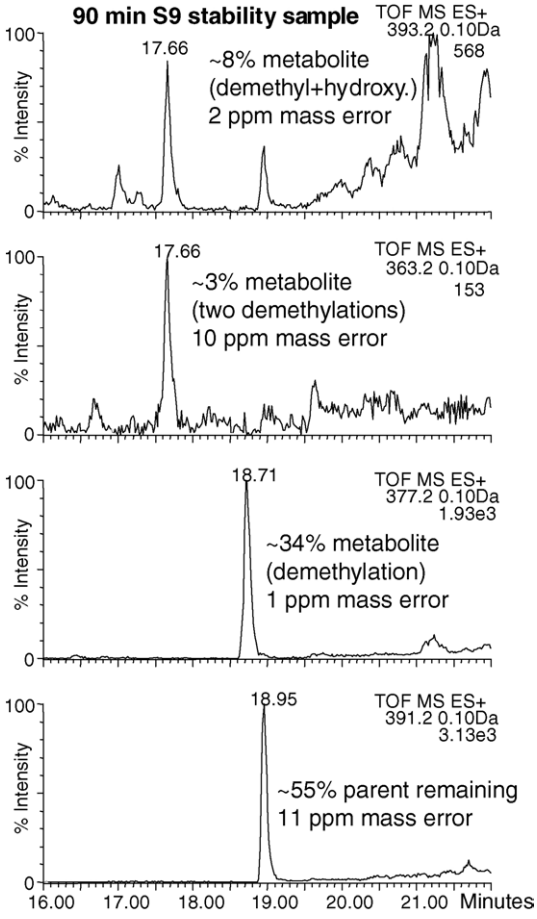

Fig. 6. Extracted ion chromatograms of the $[M+H]^+$ ions of a drug candidate and its three in vitro (rat S9) metabolites, using 0.5 mm i.d. columns for trapping and separation. Approximate relative amounts shown are based upon observed peaks. Experimental mass error for the empirical formulae of the compounds is also shown.

column and to some extent moved down the trapping column until the organic content of the injection solvent was diluted, but were then concentrated or refocused by back-flushing from the trapping onto the analytical column. This would explain the empirical observation that, generally, trapping of polar metabolites, in our hands, has been more successful with full-length (150 mm) analytical columns, as opposed to shorter cartridges.

Most importantly, Fig. 5 indicated that identifications of metabolites could be performed with the lower level incubations used for metabolic stability determinations and that a second incubation at a higher concentration was not required when using the narrow bore trapping and separation approach.

This is illustrated further with another example shown in Fig. 6. This figure shows the extracted ion chromatograms for the $[M+H]^+$ ion of an NCE and its three observed metabolites. These mass chromatograms were obtained from 3×20 μL injections of a 0.25 μM incubation of the analyte in rat S9 fraction, using matched 0.5 mm i.d. columns. Rough estimates of the relative amounts of each compound, based upon the signal from these chromatograms, are shown, along with experimental mass accuracy and the proposed identification. Adequate ion current was obtained for identification of metabolites corresponding to only 3 and 8% of the total signal. A second analysis was performed to obtain the product ion spectra of these compounds (Fig. 7). Those spectra allowed confirmation of the nature and location of the biotransformations (specific results not shown for these proprietary compounds).

Overall, trapping and separation with 0.5 or 1.0 mm i.d. columns, followed by Tof-mass spectrometric detection, has proven to be ideal for the identification of metabolites formed from in vitro incubations of NCE at 1.0 or 0.25 μM concentrations, across a range of compound classes of interest to our discovery programs. The identifications shown were readily obtained from 0.25 μM incubations, however, this in vitro screening has been more commonly performed with the higher level 1.0 μM incubations.

4. Discussion

4.1. Capillary columns

Packed capillary columns of 0.18 and 0.32 mm i.d. were also investigated (data not shown). These columns, when

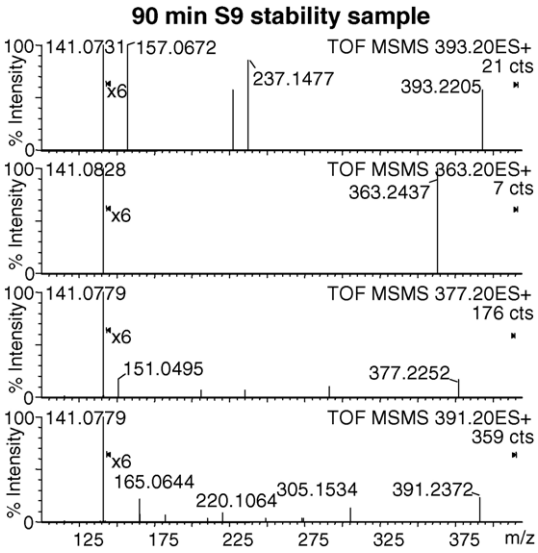

Fig. 7. Product ion spectra from the HPLC/MS/MS analysis of the sample shown in Fig. 6 using the same injection and chromatographic conditions. Although the spectra for the two low level metabolites are weak, the accurate mass assignment indicates that the signal was from the metabolites and allowed their identification.

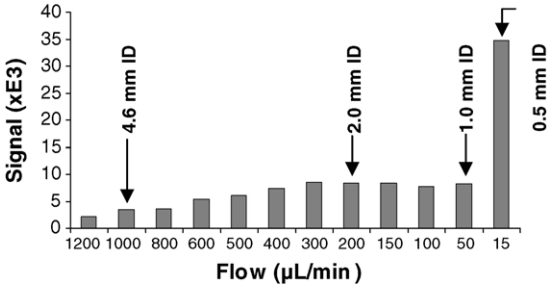

Fig. 8. Signal obtained by infusing a constant amount of analyte as a function of total flow rate into the mass spectrometer's electrospray interface. Flow rates used for different column diameters are indicated. Source conditions were optimized for each flow rate.

paired for trapping and separation, did show additional advantage in terms of concentration of analytes and utilizing even lower flow rates, resulting in improved electrospray efficiency. These advantages, however, were mitigated to some extent by additional difficulty with extra-column volumes, sample loading and decreased ruggedness and reliability. Additionally, the use of a packed capillary column for trapping, in conjunction with a narrow-bore separation column, did not exhibit the same ruggedness or show any practical peak concentration or trapping advantage over the use of a matching 0.5 or 1.0 mm i.d. column. More significantly, to obtain results from the in vivo and in vitro samples of interest to our efforts, the use of matching 0.5 or 1.0 mm i.d. columns provided adequate sensitivity, reliability and sample clean-up. The use of 0.5 mm i.d. columns has become routine for the work described here.

4.2. Low flow-rate advantage

The sensitivity advantage that is gained by the use of lower fluid flow rates with electrospray or pneumatically assisted electrospray interfaces is well-known. An illustration of this advantage, that is relevant to the narrow-bore advantages described here, is shown in Fig. 8. This graph represents the signal obtained for a constant amount of an analyte admitted to the source as a function of total flow rate introduced (signal was response of $[M + H]^+$ at m/z 569.2734). The organic content of the mobile phase was held constant at 65% acetonitrile, with 0.1% formic acid. The analyte was teed into the flowing stream before the interface and the total flow delivered included 5 μL/min from the syringe pump delivering the compound. As expected, the signal (1 min summed counts in each case) improved as the total flow into the interface decreased. Particularly interesting was that the signal spiked up significantly for the 15 μL/min experiment, the flow rate used for 0. 5 mm i.d. columns. This observation correlated generally with applied comparisons using columns with these different diameters (4.6, 2.0, 1.0 and 0.5 mm i.d.) and their associated flow rates, however, other varying factors such as on-column concentration, trapping, and impact of extra-column volume make a full-system comparison difficult. Similar graphs have been generated using different HPLC/MS instruments, with different interfaces (data not shown). While the rate of signal increase with these systems varied, as a function of flow-rate decrease, they had in common a step-function increase in sensitivity going from 50 to 15 μL/min. The sensitivity increase observed in this infusion experiment was presumably related to efficiency of the pneumatically-assisted electrospray interfacing (ionization/volatilization). This made the use of 0.5 mm i.d. columns more attractive, particularly since they were not significantly more challenging to utilize, than 1.0 mm i.d. columns, with the instrumental configuration we have described.

4.3. Sample trapping

The trapping of various polar metabolites from different matrices, in vitro or in vivo, has been most readily accomplished using a high aqueous content for the injection solvent. Likewise, washing of the trapped sample with very low organic content mobile phase to avoid loss of unknown analytes of interest has proven to be successful, as might be expected. Relative to this, there has been no issue with samples such as urine or in vitro incubates that are quenched by means other than addition of organic solvent (e.g., acid, heat). For plasma, reconstitution of protein precipitated sample has been accomplished using low amounts of organic solvent. For the in vivo example described above, the unknowns were early eluting metabolites (i.e., very polar). To successfully trap and wash these analytes, an organic content of 5% acetonitrile was utilized for both the reconstitution solvent and washing mobile phase.

In vitro incubations of NCEs for metabolic stability determinations have typically been quenched with organic solvent, presenting a sample for analysis that was only 50% aqueous. This was the case for the three examples shown in Fig. 5. In that case the analyte was retained even though 20 μL injections were used in each experiment. For the narrow-bore trapping in that situation it seems possible that at least some spreading of the analyte (or possibly even partial breakthrough) occurred. If movement on the trapping column during loading/washing does occur, the quality of the peaks obtained would seem to indicate a refocusing of the analyte during the back-flushing from the trapping onto separation column. Although satisfactory results have been obtained from 20 μL injections, as has been shown, multiple 10 μL volumes have been more commonly utilized in practical applications to minimize these issues, with compounds that are unknown at the time of the initial analysis. For samples that are highly aqueous, single injections as large as 50 μL have been used successfully onto a 0.5 mm i.d. narrow-bore trapping column.

4.4. Tof-MS advantages and general identification strategy

Time-of-flight mass spectrometry has shown several important advantages for identification of unknowns [11], particularly for work identifying trace-level metabolites in complex matrices. For acquisition of full spectrum data, the Tof mass spectrometer showed a significant sensitivity advantage over scanning instruments (e.g., quadrupoles), in both the MS and MS/MS modes (with a Q-Tof system), as would be expected. Additionally, the ability of the Tof mass analyzer to provide high resolution mass spectra allowed the determination of the mass of an unknown to within 2–10 ppm (for classical small molecule pharmaceuticals). With knowledge of the accurate mass of the starting substrate and the scope of potential biotransformations, the empirical formulae of metabolites were readily determined from an initial LC/Tof-MS analysis. The significance of this was that an initial analysis provided not only the mass of the unknown, as would be the case with nominal MS analysis (e.g., quadrupole or ion trap), but importantly the empirical formula and an assurance the analyte was drug-related. In a similar manner, with tandem quadrupole/Tof-MS analysis the empirical formulae of fragment ions from product ion scans were obtained. At a minimum this capability provided additional confidence in the identifications, but in some situations clearly provided data that would not have been readily obtained otherwise. For example, spectra from fairly weak signals provided identifications that may not have been confident except that the accurate mass analysis (i.e., empirical formulae) of those spectral peaks clearly indicated the presence of drug-related compound, as opposed to isobaric matrix components. For these reasons, Q-Tof mass spectrometry has been utilized for all our metabolite identification experiments, regardless of the separation approach utilized.

5. Conclusions

The combination of column trapping, narrow-bore chromatography, and Q-Tof mass spectrometry provides a unique capability for the identification of low level metabolites present within in vitro or in vivo samples. This combination of three well-known analytical techniques has allowed the identification of metabolites that had not been possible with related technologies. This system described here has proven to be a rugged and reliable approach that is utilized on a daily basis to provide metabolite identifications across the Discovery-to-Development continuum. Given the success of this arrangement, it seems likely that this approach would find utility for other ultra-trace identification problems.

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