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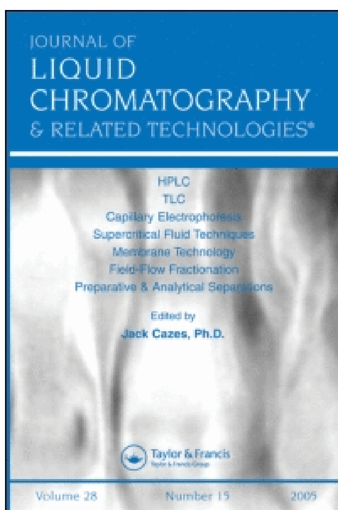

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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HIGH-THROUGHPUT MASS SPECTROMETRIC ANALYSIS OF XENOBIOTICS IN BIOLOGICAL FLUIDS

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Online publication date: 17 April 2002

To cite this Article Bakhtiar, R. , Ramos, Luis and Tse, Francis L. S.(2002) 'HIGH-THROUGHPUT MASS SPECTROMETRIC ANALYSIS OF XENOBIOTICS IN BIOLOGICAL FLUIDS', Journal of Liquid Chromatography & Related Technologies, 25: 4, 507 — 540
To link to this Article: DOI: 10.1081/JLC-120008809
URL: http://dx.doi.org/10.1081/JLC-120008809

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J. LIQ. CHROM. & REL. TECHNOL., 25(4), 507–540 (2002)

HIGH-THROUGHPUT MASS SPECTROMETRIC ANALYSIS OF XENOBIOTICS IN BIOLOGICAL FLUIDS

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ABSTRACT

The drug discovery process has been dramatically accelerated with the introduction of combinatorial chemistry for selection of new lead drug candidates. A central theme underlying this new technology is the capability to synthesize a myriad of new chemical entities with randomized structural variations. While the promise and opportunities are significant, combinatorial approaches pose several challenging tasks that must be met in order to realize the full potential of this technology. One of the challenges has been to develop fast, sensitive, and reliable high-throughput analytical methods to support investigations conducted in animals and humans. These include early pharmacokinetics screening, metabolic profiling, toxicokinetics, formulation, and eventually clinical studies. Such methods in analytical chemistry have the potential to initiate a paradigm

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transition in drug discovery landscape from rather laborious and time-consuming steps to that of an accelerated campaign for lead optimization.

Among the many intriguing avenues, mass spectrometry is arguably an indispensable analytical tool and a pragmatic approach for the identification and quantification of pharmaceutical products in biological fluids (e.g., plasma, serum, whole blood, and urine). Herein, we present a description of several laboratory procedures that have been successfully deployed to a diverse collection of issues during drug discovery and development. Techniques such as high-throughput off-line sample processing, on-line sample extraction, fast chromatographic separations, and parallel (multiplexed) liquid chromatography in conjunction with mass spectrometry are discussed.

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INTRODUCTION

Since the evolution of pharmaceutical research, (1) the stages of drug discovery and development have followed three predominant routes: (i) the systematic and methodical approach by chemists to design and synthesize a molecule to target a specific molecular system (e.g., ion-channels, receptors, enzymes, DNA); (ii) the isolation and purification of the active ingredients of medicinal plants or microorganisms and to screen their spectrum of activity using *in vitro* models; or, (iii) the serendipitous discovery of a compound with a novel pharmacological action (e.g., the accidental discovery of antidepressants). Today, one of the increasingly popular and complementary approaches for drug discovery in pharmaceutical industry is to perform massively parallel synthesis in solution or on a solid support. In addition, with the advent of functional genomics and proteomics, cell-based assays, and molecular biology, a multitude of therapeutic targets has been validated (2–9).

With an increasing number of potential molecular targets identified through the science of functional proteomics and genomics, diverse libraries of new chemical entities (NCE) have to be generated and evaluated. Consequently, the rapid growth of combinatorial libraries has posed a need for faster, accurate, and sensitive analytical techniques capable of large-scale high-throughput screening (HTS). Although *in vitro* assays do not necessarily reflect the complexity of the *in vivo* interactions; due to their speed and simplicity, these assays have become an integral part of the screening process. Furthermore, samples generated from large scale clinical trials along with the ambitious development timelines warrant the use of high-throughput bioanalysis. Numerous improvements in speed, sensitivity, and accuracy, augmented with innovations in automation place mass

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spectrometry (MS) among one of the versatile and multi-faceted analytical techniques available today.

Toward this end, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been valuable for both qualitative and quantitative screening of small molecules (e.g., pharmaceutical products). This article is intended to discuss a brief overview of techniques involving high-throughput mass spectrometry (HT-MS) in conjunction with some of the typical problems presented to the analytical pharmaceutical chemist.

Due to space limitations, we can only present what we perceive to be the most significant and recent applications for rapid quantitative analysis of small molecules in biological fluids. In addition, it is our view that the majority of drug discovery efforts by the pharmaceutical industry are likely to focus on small molecule agents. For detailed technical descriptions and additional applications, the reader is referred to the corresponding citation(s) throughout this manuscript.

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IONIZATION PROCESSES

MS is playing an increasingly visible role in the molecular characterization of combinatorial libraries, (10–12) natural products, (13) drug metabolism and pharmacokinetics, (14–18) toxicology and forensic investigations, (19) and proteomics (20–26).

The utility of ESI lies in its ability to generate ions directly from the solution phase into the gas phase. The ions are produced by application of a strong electric field to a very fine spray of the solution containing the analyte. The electric field creates highly charged droplets whose subsequent vaporization (or desolvation) results in the production of gaseous ions (27,28). The fact that ions are formed from solution has established the technique as a convenient mass detector for liquid chromatography (LC/MS) and for automated sample analysis. In addition, ESI-MS offers many tangible benefits over other mass spectrometric methods including the ability to qualitatively analyze low molecular weight compounds, inherent soft-ionization, excellent quantitation and reproducibility, high sensitivity, and amenability to automation.

Analogous to the ESI interface, atmospheric pressure chemical ionization (APCI) or heated nebulizer (HN) induces little or no fragmentation to the analyte. Therefore, the APCI spectrum also tends to be simpler in interpretation than the traditional electron ionization (EI). Generally, volatile and thermally stable compounds can be subjected to LC/APCI/MS analysis. In quantitative analysis, APCI provides a higher (i.e., in terms of linearity) dynamic range than ESI and it is considered rugged, easy to operate, and relatively more tolerant of higher buffer concentrations (i.e., fewer matrix effects). In ESI, at about 10^{-5} M and higher, the

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ion signal becomes fixed and independent of sample concentration (plateauing effect) and may exhibit non-linearity at higher concentrations (29,30). In contrast, APCI can offer a wider linear dynamic range. For example, in our laboratory (data not shown) we have routinely developed and validated racemic reversed-phase LC/APCI/MS/MS assays ranging from 1.0 ng/mL to 10,000 ng/mL with a correlation coefficient of > 0.996. Furthermore, APCI can accommodate flow rates of up to 2.0 mL/min and, thus, it is considered mass sensitive as opposed to ESI, which is concentration sensitive. In contrast to ESI, APCI is not suited for the analysis of biopolymers and thermally labile species.

In the APCI process, electrons originating from a corona discharge needle (with a typical current between 1–5 μAmp) ionize the analyte *via* a series of gas-phase ion-molecule reactions. For example, in the positive-ion mode, the energetic electrons start a sequence of reactions with the nebulizing gas (typically nitrogen) giving rise to nitrogen molecular ions (28). Depending on the composition of the HPLC mobile phase, ions such as $[H_2O + H]^+$, $[CH_3OH + H]^+$, $[NH_3 + H]^+$, and/or $[CH_3\ CN + H]^+$ are formed *via* series of ion-molecule reactions with the nitrogen molecular ions. Subsequently, additional ionization is initiated by exothermic proton transfers from the protonated solvent ions to the neutral analyte molecules, yielding $[\text{analyte} + H]^+$, $[\text{analyte} + CH_3OH + H]^+$, $[\text{analyte} + NH_3 + H]^+$ ions, etc. Greater sensitivity is attained if the solvent is polar and contains ions through the addition of an electrolyte. The desolvation process is then further enhanced by the heating element within the APCI assembly, which is maintained at 300–550°C. Since the direct introduction of higher HPLC flow rates (e.g., 1.0 mL/min for a 4.6 mm, i.d. column) is amenable to APCI, chromatographic dead volume is less of an issue (31).

In general, because the chromatographic concentration increases as an inverse function of the square of the column diameter, lower i.d. HPLC columns could be employed in quantitative trace analysis (e.g., $\leq$10 pg/mL). One of the drawbacks of APCI is its lack of compatibility with 1.0, 0.50, 0.32, and 0.18 mm, i.d. columns with typical respective flow rates of 50, 15, 6, and 2 μL/min. Therefore, with a mass sensitive device such as APCI, no sensitivity gains are realized with smaller columns or flow rates. In contrast, ESI is compatible with miniaturized columns and amenable to sample-limited scenarios such as biochemical and biotechnological applications (31,32).

HIGH-THROUGHPUT OFF-LINE SAMPLE PROCESSING FOR QUANTITATIVE ANALYSIS

One of the critical steps in qualitative and quantitative analysis is the sample processing procedure. Sample preparation steps can affect specificity,

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sensitivity, accuracy, precision, and throughput of a bioanalytical procedure. In addition to development and optimization of the chemistry involved in sample processing, the use of semi-automated or fully automated protocols has been implemented in recent years (18). The popularity of off-line sample processing in batch-mode has dramatically improved the throughput of this rate-limiting step.

Generally, there are three commonly used approaches for off-line sample processing: solid-phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PPT). All the above methods have been successfully used in conjunction with robotics for achieving an increase in sample preparation throughput. For example, Figure 1 depicts a schematic representation of a semi-automated sample preparation that can accommodate SPE, LLE, or PPT procedures. This scheme has been validated for use with SPE, LLE, or PPT in a 96-well plate format for the analysis of pharmaceutical products in biological matrices (e.g., whole blood, plasma, serum, and cerebral spinal fluid) in our laboratory.

In the 96-well SPE format, similar to the traditional manual procedure, issues such as the nature of the bonded-phase (e.g., mixed phase cation exchange, C_{18}, C_8, etc.), solvent strength to condition, wash, elute, and chemical characteristics (e.g., solubility, presence of the key functional groups, etc.) of the analyte(s) need to be addressed. Some of the most commonly utilized robotic modules for the 96-well SPE procedure are Tomtec Quadra (Tomtec, Hamden, CT, USA), Packard Multi-Probe (Packard Instruments, Meriden, CT, USA), and Tecan (Durham, NC, USA) units. For example, Tomtec Quadra has been successfully adopted in the development and validation of several off-line SPE assays in whole blood, (33) plasma, (34–36) and urine (37,38) followed by MS detection. Likewise, the feasibility of a Tecan system for the quantification of a neurokinin-1 (NK-1) antagonist in clinical studies was reported by Schmid and co-workers. (39) A lower limit of quantification (LLOQ) of 0.10 ng/mL was attained using a LC/ESI/MS/MS method.

The Packard Multi-Probe liquid handling workstation has also shown promise for off-line SPE procedures involving plasma (40–42) and serum (43,44). In addition, this unit, as well as the Tecan system, can be programmed for the initial sample transfer step from vials to the 96-well blocks, buffer addition (if applicable), and to aliquot internal standard. The implication of the above capabilities is a significant reduction of time and labor, which is required for the entire sample processing procedure. Possible technical problems, such as carry-over by fixed tip pipettes used to aliquot the biological fluid, can be alleviated by incorporation of several wash cycles or their replacement with disposable pipette tips. In addition, possible inaccurate transfer of samples from the collection tubes to the 96-well blocks due to pipette tip clogging by endogenous protein clots or lipid layers should be kept in mind.

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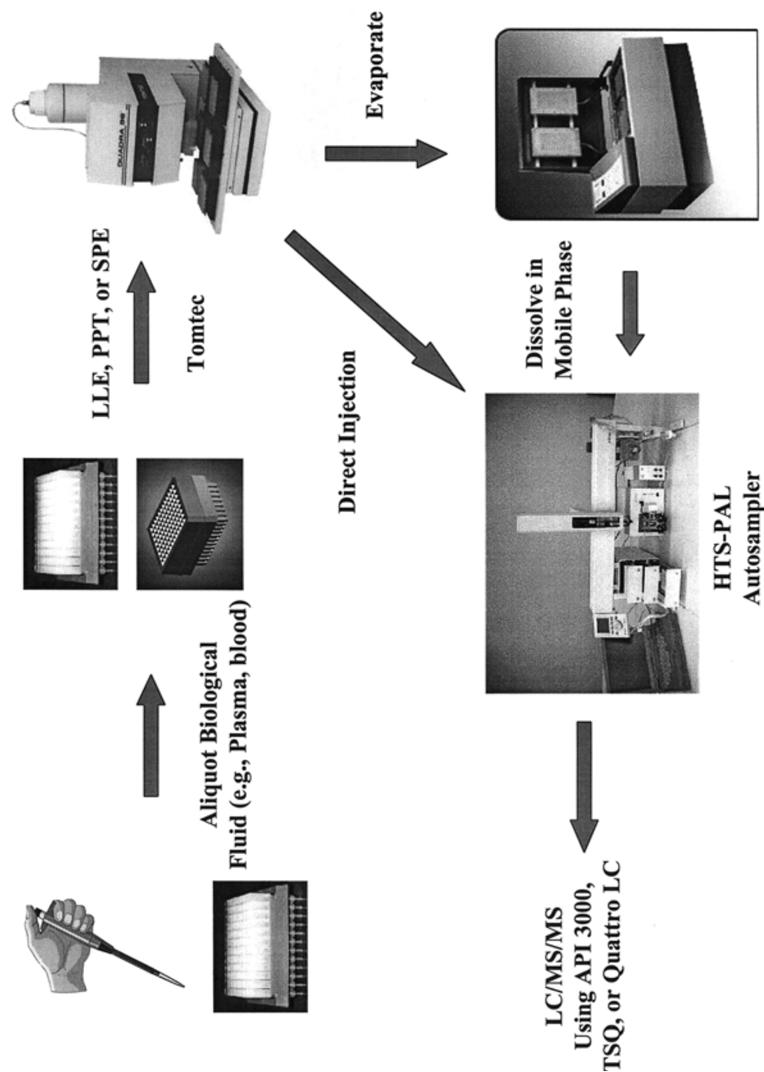

Figure 1. A schematic representation of the semi-automated sample preparation.

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Recently, Watt and co-workers (45) proposed the storage of the plasma samples at $-80^{\circ}C$ and thus precluding fibrinogen clot formation. A centrifugation step at 14,000 rpm prior to sample transfer has been suggested by Rossi and co-workers (46). Recently, the application of higher density plate formats such as 384-well SPE has been realized (47,48). While higher density plates (e.g., 384- and 1536-well formats) can dramatically increase sample throughput, there are several technical caveats that warrant close scrutiny prior to their universal adaptation in conjunction with LC/MS technology. First, the modification of exiting 96-well format laboratory technology to higher density plates should be considered. Second, higher density plates can accommodate smaller volumes of sample, which may require the assay to be run at lower LLOQ. A transition to smaller internal diameter columns (e.g., 1.0 mm, capillary LC) is an option to attain lower LLOQ for trace quantitative analysis. However, columns with smaller diameters tend to be less rugged when subjected to large number of injections of biological extracts. Third, re-injection of a sample (that is not uncommon during routine analysis) may not be viable due to the lack of adequate volume. We envision all the above issues to be resolved in the near future.

Liquid–liquid extraction is another well-established and attractive approach, which has been useful for the analysis of xenobiotics in biological fluids. LLE can be designed to be highly selective yielding clean sample extracts. This is particularly critical in minimizing ion-suppression by co-eluting matrix components, when an ESI interface (it appears that ion-suppression is not a major determinant for signal loss in APCI) is used for the LC/MS analysis (49–52). The ion-suppression is exacerbated in cases where fast chromatography results in low peak capacity factors (e.g., $k' \leq 2$). Based on a series of experiments reported by King and co-workers, the order of ESI response suppression is PPT > SPE > LLE, where liquid-liquid extraction leads to the least amount of analyte ion loss (53). Till 1999, the number of reported semi-automated LLE procedures was scarce. However, since then, there has been an increasing number of articles published in this area (54–65).

Recently, we described a HT sample preparation procedure in conjunction with racemic LC/APCI/MS/MS analysis for methylphendiate (MPH), a dopamine transporter inhibitor, with a LLOQ of 50 pg/mL (65). A semi-automated robotic method using liquid-liquid extraction (LLE) in a 96-well plate format was developed and validated. The correlation coefficients were ≥ 0.998 for MPH indicating good fits of the regression models over the range of the calibration curves.

Similarly, we applied the same LLE approach for the analysis of MPH enantiomers in human plasma using a reversed-phase chiral LC/APCI/MS/MS analysis. Vancomycin based chiral stationary phase was used to separate the *d*-(the pharmacologically active antipode) and *l*-MPH under 7.5 min. The LLE

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yielded clean sample extracts and aided in the longevity of the chiral column. Consequently, the vancomycin-bonded stationary phase exhibited excellent performance with no separation deterioration observed after ~2500 injections per column. Figures 2 and 3 show the representative LC/APCI/MS/MS ion-chromatograms obtained for the MPH and its stable isotope internal standard for the racemic and the chiral assays, respectively. Both methods were routinely used in the quantification of MPH or its enantiomers in several toxicokinetic and clinical studies. For example, Figure 4 depicts a plot for the dose-normalized area under the curve (AUC), subsequent to 13 weeks of daily oral administration of 80 mg/kg of racemic MPH in female and male rats. The HT-LLE method followed by chiral chromatography-tandem mass spectrometry clearly revealed gender specificity with respect to MPH exposure. Female rats in general showed higher exposure of MPH than the males. Regardless of the gender, rats dosed with MPH racemate (80 mg/kg/day) exhibited ~2–3 times higher concentrations of the *d*- than the *l*-isomer.

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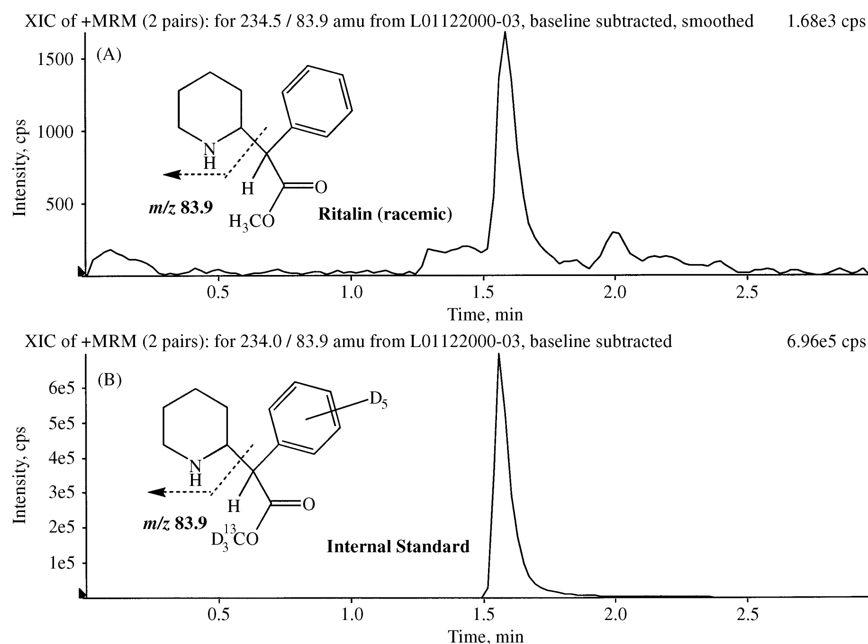

Figure 2. MRM-chromatograms resulting from the analysis of 0.050 ng/mL of MPH and internal standard (injection volume: 40 µL). Panels A and B correspond to MPH (MRM transition: m/z 234.5 → 83.9) and the internal standard (MRM transition: m/z 243.0 → 83.9), respectively. Molecular structures of both compounds are shown.

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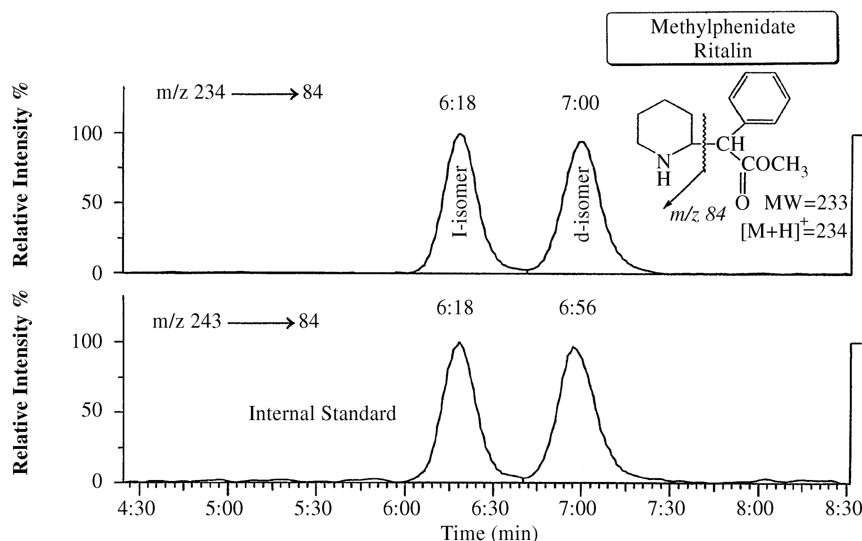

Figure 3. Representative LC/APCI/MS/MS ion-chromatograms resulting from the analysis of 2.18 ng/mL of racemic standard + internal standard (injection volume: 30 μL). Panels A and B correspond to the MPH and its internal standard, respectively. The concentration of the ammonium trifluoroacetate (TFAA) was 0.05% (by weight). The HPLC flow rate was 1.0 mL/min.

Protein precipitation using acetonitrile is one of the commonly used procedures for treatment of plasma samples in drug analysis. Recently, several groups reported the application of PPT using high-throughput bioanalysis protocols in conjunction with LC/MS/MS detection (45,66–68).

Briefly, an accurate amount of each sample is transferred from the collection tube to a 96-well block. The sample is spiked with the internal standard and vortexed. An appropriate volume of the precipitation solvent (the volume and composition of the precipitation solvent is optimized by the analyst) is added to the sample, mixed, and centrifuged for 10–15 min. The speed of the mixer is adjusted to prevent any possible leakage between the wells. Subsequent to centrifugation, a multi-channel pipettor is then utilized to transfer the supernatant layer into another clean 96-fighting fit block without disturbing the protein pellet. The samples can be either directly injected for quantification, or evaporated and reconstituted with the appropriate mobile phase prior to LC/MS analysis (Figure 1).

An alternative to sample centrifugation in PPT is sample filtration in a 96-well plate format (69,70). This step can aid in the removal of the larger particles

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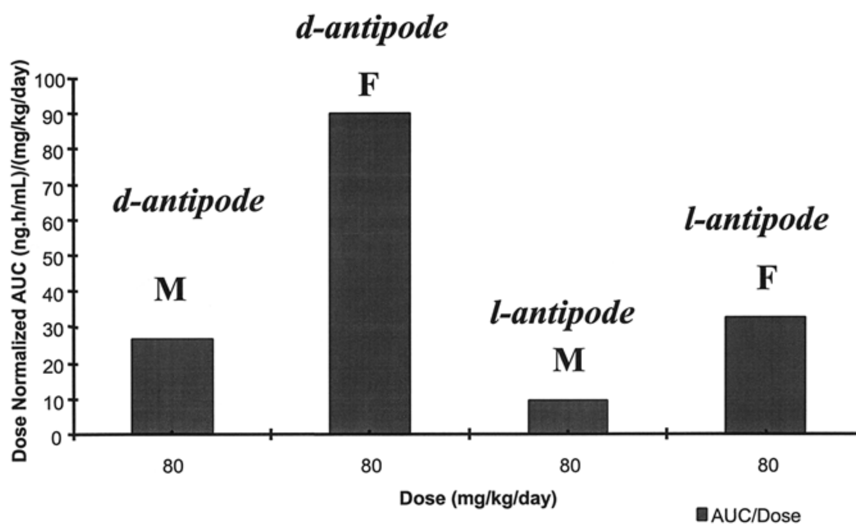

Figure 4. Mean dose normalized AUC (ng.h/mL)/(mg/kg/day) versus dose (mg/kg/day) profiles of rat plasma *d*-MPH and *l*-MPH subsequent to daily oral administration of 80 mg/kg of racemic MPH for 13 weeks.

(e.g., protein aggregates, particle sizes $\geq 10\,\mu m$) that may otherwise render technical difficulties during analysis. Filters such as 3M Empore™ (part number: 6060; 3M, Minneapolis, MN, USA) or UNI-FILTER GF/B glass fiber (part number: 7700-1803; Whatman, Clifton, NJ, USA) 96-well plates are among a number of options available for protein filtration. The feasibility of 96-well small volume PPT to remove precipitated proteins, thereby eliminating the centrifugation step, has been described (70).

As mentioned earlier, ESI signal suppression due to co-eluting matrix components in supernatants (53) can compromise the sensitivity, accuracy, and precision of the assay during validation. Thus, APCI or heated nebulizer is the preferred choice to alleviate such a problem. However, APCI will not be amenable to thermally labile analytes (71). Alternatively, longer isocratic elution chromatography (higher peak capacity factor) or gradient elution LC can be adopted to separate the endogenous matrix components from the analyte(s) of interest (49). Ultra-fast reversed-phase HPLC in isocratic or gradient elution modes has been gaining popularity for rapidly (i.e., a shorter LC/MS injection-to-injection cycle) achieving sufficient chromatographic resolution (72–81). Various column technologies such as nonporous, porous ultra-microparticles ($\leq 2\,\mu m$), monoliths, and superficially porous particles ($\sim 5\,\mu m$) offer specific advantages and disadvantages (82). For example, Figure 5 shows representative

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LC/APCI/MS/MS ion-chromatograms resulting from the analysis of 2.0 ng/mL (LLOQ) of ketoconazole and its internal standard. Panels A and B correspond to the ketoconazole and the internal standard, respectively. A column with a continuously tapered bore over its 25 mm length from 2.0 mm (i.d.) at the inlet to 0.50 mm (i.d.) at the outlet was utilized (68). According to the manufacturer, the column used in our study has a dead volume of ca. 40 μL. Incorporating the flow rate of 0.40 mL/min, an estimated t_o (column dead time) of 10 s is obtained. Therefore, based on the retention time of ketoconazole ($t_R \approx 17$ s), a retention or capacity factor (k') of ~0.70 can be estimated for the ion-chromatogram shown in Figure 5. (68)

Several examples on application of HT-enantioselective liquid chromatography-mass spectrometry in analysis of chiral pharmaceutical products have been described (83). In spite of the MS/MS selectivity, it is important to keep in

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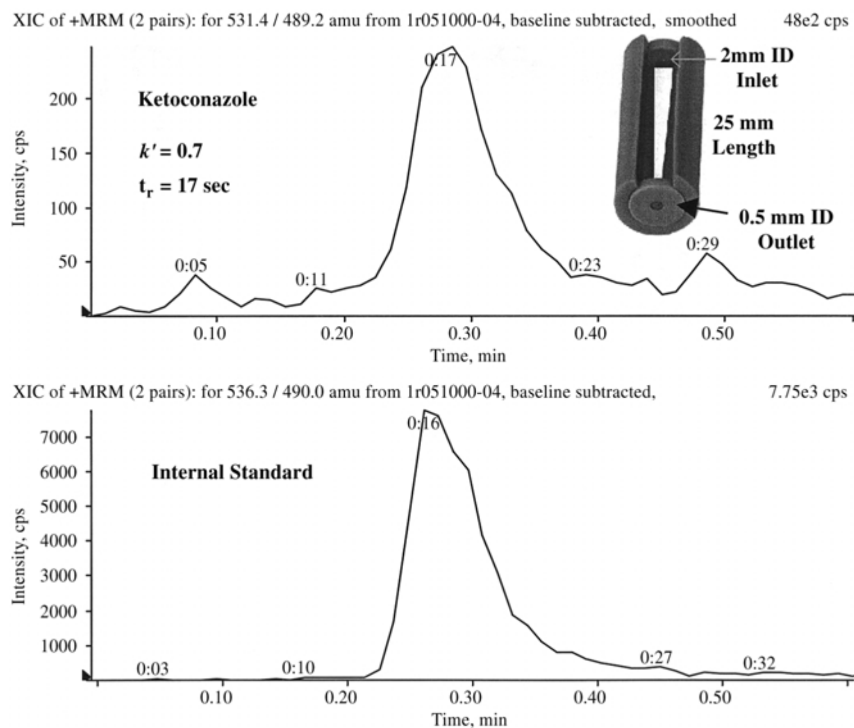

Figure 5. Representative LC/APCI/MS/MS ion-chromatograms resulting from the analysis of 2.0 ng/mL (LLOQ) of ketoconazole + internal standard (using the Michrom Magic Bullet^{TM} Column; injection volume: 10 μl). Panels A and B correspond to the ketoconazole and the internal standard, respectively.

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mind that fast turn-around LC/MS/MS assays can be prone to interference. For example, during the analysis of *in vivo* samples, the absence of adequate chromatography (i.e., co-elution of analyte and possible metabolites) coupled to MS detection may yield interference, which is not easily detected during the validation process. Recently, Jemal and Xia (84) demonstrated the possibility of in-source dissociation of biotransformation products yielding the parent compound. Therefore, false positive values in terms of *post-dose* analyte(s) concentrations, which could stem from the in-source dissociation of the putative metabolite(s) yielding identical MS/MS transitions, can result. Hence, during the analysis of post-dose biological samples, a clear understanding of the identity and abundance of *in vivo* biotransformation products is essential.

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HIGH-THROUGHPUT ON-LINE SAMPLE PROCESSING FOR QUANTITATIVE ANALYSIS

In recent years, high-throughput and automated on-line sample extraction approaches have provided viable alternatives to improve efficiency for sample processing. We have capitalized on this approach, and reported the utility of an on-line turbulent flow chromatography (85–88) unit coupled to MS for the quantitative analysis of terbinafine (89) and ketoconazole (68) in human plasma.

According to the well-established mathematical approximation by van Deemter, LC optimization can be achieved by reducing zone broadening and/or altering relative migration rates of the analytes. At relatively low mobile phase flow rates (i.e., 0.5–1.0 mL/min for 4.6 mm, i.d.), optimum chromatographic separation and efficiency may be achieved. The efficiency is, in part, governed by the mass transfer rate for solute molecules to diffuse in and out of the stationary phase. Furthermore, a majority of chromatographic separations are conducted under laminar flow, where the flow profile of the mobile phase has a parabolic shape and, consequently, solutes at the front of the band exhibit a mass transfer rate different from that of the solutes at the trailing edges. However, in turbulent flow using large particle diameters (to reduce column back-pressure), the solvent front exhibits a plug nature rather than a parabolic shape. The combination of large particle size and higher flow rate results in eddies which facilitate solute distribution and equilibration into the stationary phase (e.g., an increase in diffusion rate). Thus, the net effect of turbulent flow chromatography is a flow regime beyond the van Deemter approximation.

In turbulent flow LC, single- and dual-column configurations have commonly been reported. In both cases, an extraction column of various functionalities and particle size (30 μm in diameter or higher) are used. A typical dimension of the extraction column can be 50 × 1.0 mm (i.d.), although smaller lengths can also be attempted. In the single-column configuration, a sample

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containing the analyte and internal standard is loaded on the extraction column at a high linear velocity (e.g., 5.0 mL/min). The analyte is retained *via* rapid diffusion into the packing, while other matrix components are washed into waste using an aqueous mobile phase. Subsequently, the analyte is eluted by a step or linear gradient and detected by the mass spectrometer. In the dual-column configuration, a standard analytical column (e.g., C_{18} or C_8; 50×4.6 mm, i.d.) is placed after the extraction column to improve chromatographic separation and sample clean-up. In our laboratory, we have successfully validated and applied the dual-column configuration (Figures 6 and 7) to perform racemic reversed-phase (68,89) as well as chiral LC/MS/MS analysis. In the latter assay, we replaced the second column by one containing a chiral stationary phase. A full account of the assay optimization and validation of an on-line achiral-chiral column configuration will be reported elsewhere.

Direct sample injections have also been accomplished by using on-line C_{18} (4 mm, i.d.) guard cartridges for cytochrome inhibition studies (90–92), capillary SPE(93), C_{18}-alkyl-diol-silica restricted access phase (94), and PROSPEKT^{TM} SPE modules (95–98). A caveat for all the direct sample injection assays is an understanding of the analyte stability in the biofluid during the analysis period. Nonetheless, an increasingly growing body of literature (99–107) is suggestive that direct injection of post-dose biological fluids for quantification purposes has become a routine and efficient procedure.

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APPLICATIONS OF PARALLEL LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

An additional enhancement in the throughput of an LC/MS method is achieved by devising a parallel loading or multiplexed experiment (Figure 8). In this design, a four- or eight-channel multiplexed configuration is used to analyze multiple sample streams on a single mass spectrometer. For example, in the four-channel configuration, a pump, a splitter, and individual injector valves are adjusted to deliver equal mobile phase flow rates to each column. It is crucial to maintain the same type of guard columns, HPLC columns, and plumbing for all channels to ensure nearly equal back-pressures. The effluents from four identical HPLC columns are then directed to each corresponding UV detector. The UV detector output provides additional information on the purity of the sample (e.g., qualitative analysis), but it is not an essential component of the unit for quantitative analysis. The effluent from each individual channel is then introduced to a splitter, and a fraction (e.g., 50–100 μL/min) of it is directed to the MS ion source. The MS ion source known as MUX^{TM} (Micromass UK Limited, Manchester, England) consists of an ESI with four- (Figure 9) or

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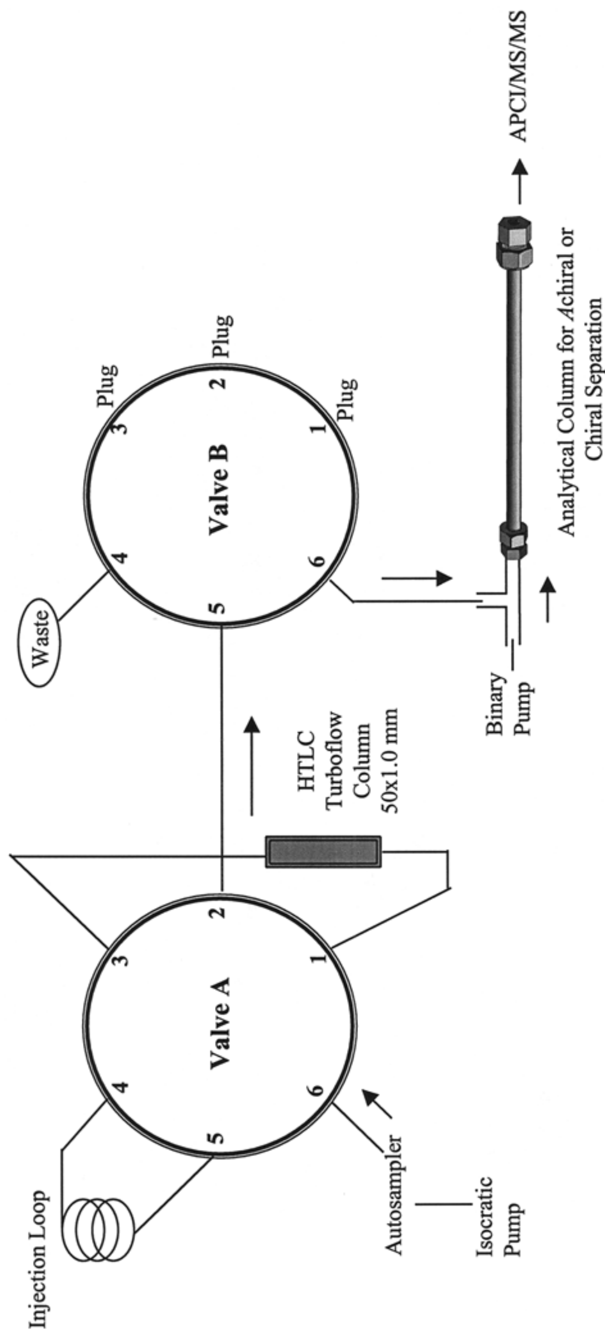

Figure 6. Simplified schematic of 2-valve/2-column system used in the turbulent flow LC experiment. The first column (50 × 1.0 mm) is used for the extraction step.

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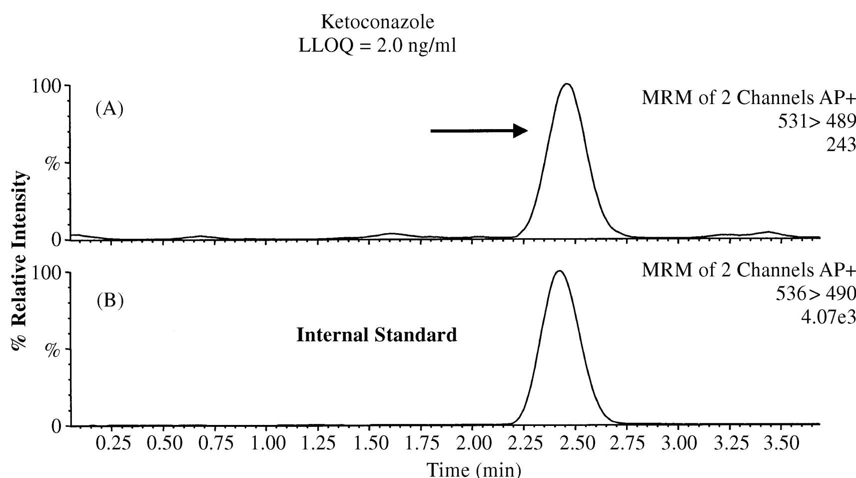

Figure 7. Representative LC/APCI/MS/MS ion-chromatograms resulting from the analysis of 2.0 ng/mL (LLOQ) of ketoconazole + internal standard (using the turbulent flow LC system; injection volume: 20 µl). Panels A and B correspond to the ketoconazole and the internal standard, respectively.

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eight-sprays. A co-axially positioned cylinder and stepper motor allows only one of the sprayers to be sampled by the mass spectrometer at any given time. For this system to function properly, all the above hardware components need to be operative in an integrated and synchronous fashion. The minimum duration needed to step from one sprayer to another (inter-spray step or switching time) is 50 ms. The minimum dwell time for each channel is about 50–100 ms. The total cycle time is about 1.2 s. Generally, a time-of-flight (ToF) or triple quadrupole MS is used for detection. The latter is commonly interfaced to a four-channel configuration. However, due to a high acquisition rate offered by ToF analyzers (>10 spectra/s), this instrument is available for use with either configuration. The triple quadruple mass spectrometer offers a wider dynamic range than the ToF instrument for quantitative analysis.

Currently, the triple quadrupole analyzers are the most popular and rugged instruments for quantitative analysis when used in the multiple-reaction-monitoring (or selected-reaction-monitoring) mode. On the other hand, ToF analyzers are capable of obtaining good quality spectra with moderate to high resolution, accurate mass measurements, fast scanning ability, and is suitable for qualitative analysis. Regardless of the detector choice, it is highly recommended that the possibility of any sample carry-over to be assessed (this is particularly important at high concentrations). Recently, several groups have reported

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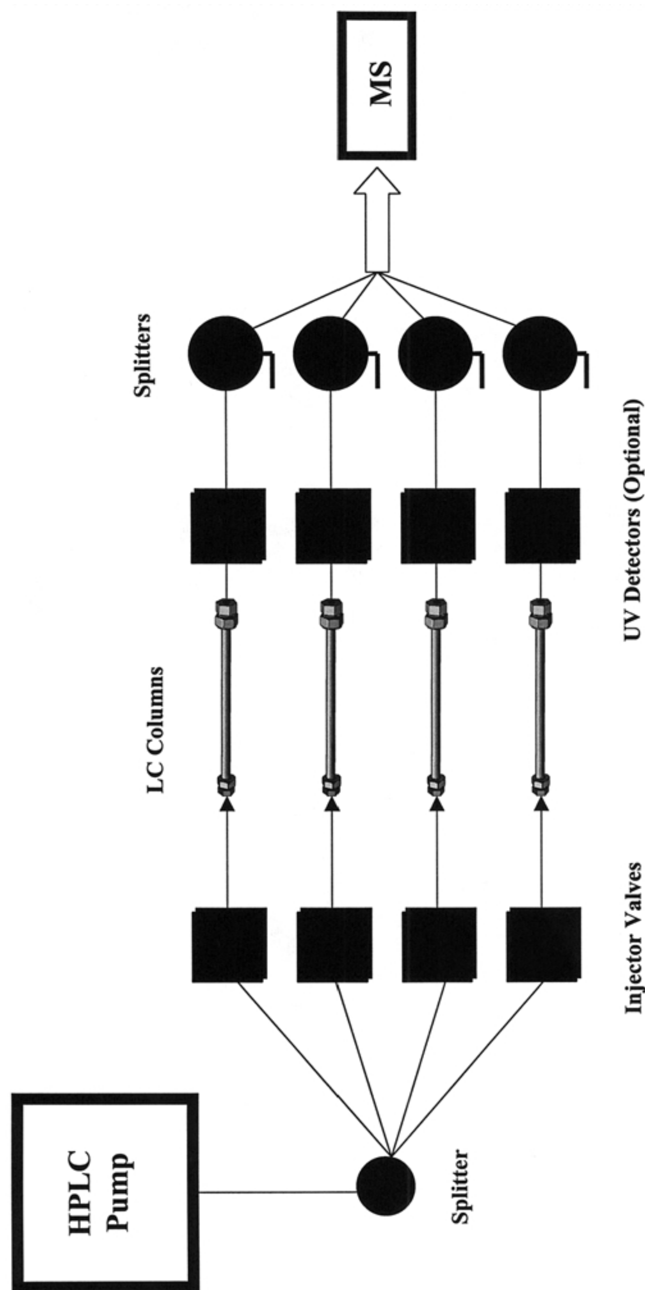

Figure 8. Simplified experimental set-up of a four-channel parallel (multiplexed) electrospray interface unit.

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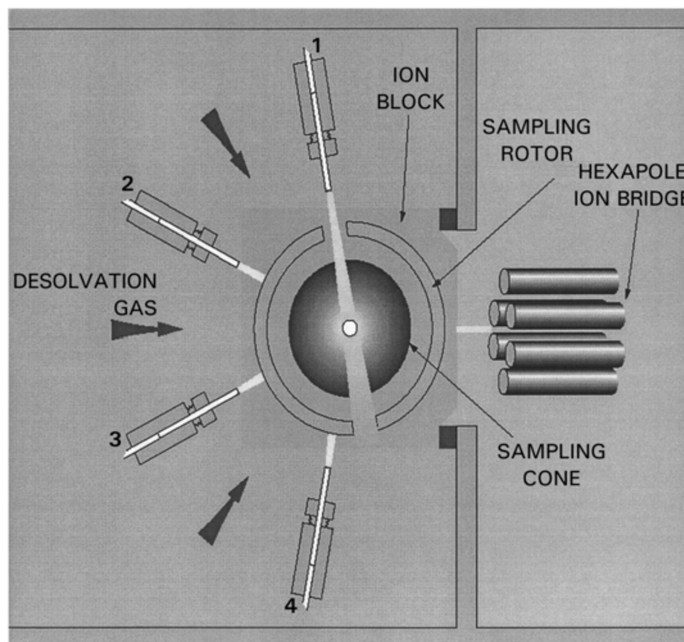

Figure 9. Schematic diagram of a four-channel parallel electrospray interface ion source or MUX^{TM}.

(108–112) the feasibility of parallel LC/MS in conjunction with the MUX^{TM} technology. It is evident that the parallel LC/MS approach can increase the throughput by a factor of 4 and 8 for the four- and eight-channel configurations, respectively.

In addition to the multiple sprayer technology (*vide supra*), a host of other clever bioanalytical approaches have been reported (113–120) to gain an increase in sample throughput. Janiszewski and co-workers (113) devised a multiple injector-dual column configuration module using one autosampler to perform rapid metabolic screening of samples obtained from caco-2 and hepatocyte incubations. Jemal et al. (114) measured the plasma concentration of nefazodone, a selective 5-HT_2 antagonist, and its three metabolites using a dual-autosampler/dual-column system with a single mass spectrometer. Synchronized multiple-autosampler units coupled to a single HPLC column (115) or multiple-column configuration (116,117) can be easily retrofitted to an existing mass spectrometer. In addition, multiplexed designs allow adequate chromatographic separation (if needed) without sacrificing efficiency (114).

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The multiplexed or parallel strategy has also been incorporated into the on-line extraction assays (118–120). Generally, when the analyte from the first injection is eluted and detected by the MS, a valve is switched to the second column for a similar measurement. In the meantime, the first set of extraction and analytical columns is washed, equilibrated, and loaded with the biofluid (e.g., plasma). This cycle of staggered parallel separation is also referred to as the ternary-column system (118).

THE FEASIBILITY OF AN AUTOMATED BLOOD SAMPLING SYSTEM IN COMBINATION WITH HT-MS

In spite of the fact that the above technologies can increase efficiency during sample preparation, the initial transfer of sample (e.g., plasma) aliquot from the collection tube to the 96-well plate remains to be the bottleneck. In our laboratory, the process of manual uncapping, transfer of sample aliquot, and capping the vial requires about 20–30 min for each 96-well plate. In addition, manual blood collection after regular business hours can be a limiting factor. Recently, we described a preliminary study on the feasibility of an automated blood sampling system (Culex) in conjunction with LC/MS/MS analysis for the quantification of ketoconazole in rat, subsequent to an oral dose of 10 mg/kg of the test article (121). The ketoconazole assay was a validated semi-automated method involving PPT in 96-well format to attain high-throughput sample preparation (68).

Figure 10 shows the front views of the Culex unit and its animal housing. One of the unique features of this system is the ability of the rat to freely move within the cage while attached to the catheter line. In this design, the catheter line is connected to a counter balance arm with sensor, which triggers the cage's turntable to rotate in an opposite direction to the rat movement. This action results in maintaining the structural integrity of the catheter line without twisting and stretching. The metabolic cage is also capable of urine and feces collection in a separate fashion that remains out of the reach of the animal and any possible cross contamination. The urine is funneled *via* stainless steel mesh to a glass vial maintained at $\leq 4^{\circ}$C. The feces are diverted down through a steep incline, stored outside the metabolic cages, and separated from the urine collection path.

The drawbacks of the above particular design include its lack of feasibility to studies involving larger animals (e.g., dog, and monkey). Such studies could be amenable if the subject is tethered or restrained in some fashion (e.g., a primate in a chair). An alternative approach involves the use of robotics (e.g., Packcard Multi-Probe) for the direct transfer of plasma or serum to the 96-well plate. Furthermore, currently, a fully-loaded Culex system will house up to four animals (e.g., 1 rat/metabolic cage) which makes it unsuitable for larger scale studies.

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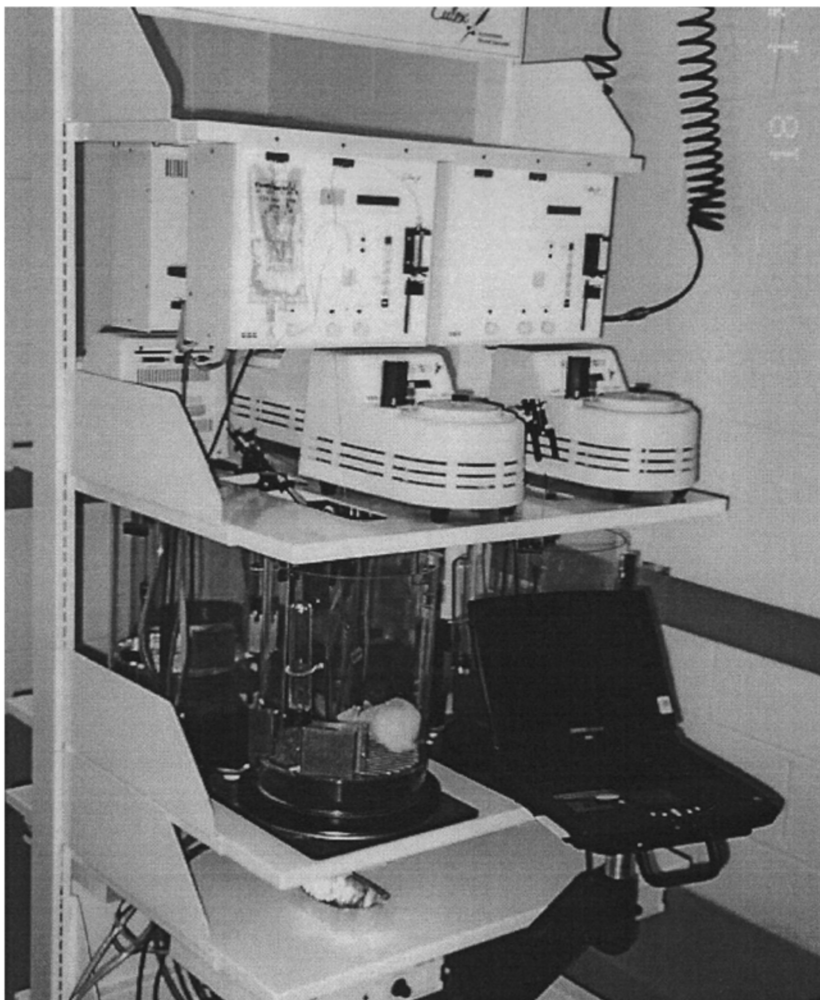

Figure 10. Front view of the Culex unit. This particular unit accommodates up to 4 rats (one rat per cage) and collects the blood into refrigerated vials that can be transferred to a 96-well plate.

Although a costly approach, the latter issue can be addressed by using several Culex units.

The Culex system has the potential to be integrated into the so-called "cassette," "N-in-one," or "cocktail" dosing pharmacokinetic studies, at the early

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stages of drug discovery (122–124). In these studies, structurally analogous classes of drug candidates are administered simultaneously in a laboratory animal; and consequently, the estimated AUC values are calculated and compounds are prioritized. The "N-in-one" dosing experiment requires smaller number of animals and is more efficient than the traditional one compound per dose per animal. Several caveats that accompany the above approach, include an increase in LC/MS method development and data analysis and possible drug-drug interactions yielding false-positives and distortion of pharmacokinetic profiles (8). For example, inhibition of metabolism can occur when drugs compete for the same metabolic pathways and, thus, elevated AUC levels may be observed. Hence, alternative routes can be taken which include single-dosing/multiple-analysis and "sample-pooling" to expedite pharmacokinetic measurements (125,126).

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CONCLUSIONS AND FUTURE PROSPECTS

A myriad of published reports has now proven the broad applications of modern MS-based techniques to the analysis of small molecules. While the focus of this article was quantitative analysis, the reader should bear in mind that LC/MS and tandem MS techniques are also utilized as sensitive and robust analytical tools in qualitative measurement of xenobiotics in biological fluids. While in the spectrophotometric detection, potential for interference from endogenous species and mobile phase additives may exist, the MS selectivity prevails for trace qualitative analysis.

The significance of early PK studies has been well established among the pharmaceutical industry (127). The major determinants of PK parameters are absorption, distribution, metabolism, and excretion (ADME). Toward this end, qualitative mass spectrometry has enabled medicinal chemists to address issues such as metabolic stability and modification of metabolically vulnerable moieties (soft-sites) of the lead drug candidates, in order to optimize their PK and pharmacodynamic attributes (128). Normally, several iterations between drug metabolism scientists and synthetic chemists are required to link and improve these molecular properties (129).

Techniques, such as automated data-dependent tandem mass spectrometry, will continue to be a powerful tool for acquiring simultaneous full scan MS and MS/MS within a single chromatographic run (130–132). In this mode of operation, the instrument collects full scan MS spectra and switches to tandem MS mode when a preset ion threshold is exceeded. This cycle continues till the end of the HPLC run and can provide molecular weight and in-depth structural information from a single injection.

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In contrast to quantitative analysis, which often entails the analysis of large quantities of samples, structural elucidation involves a smaller number of samples. However, the rate-limiting step in qualitative mass spectrometry has been the interpretation of MS and tandem mass spectrometry data obtained from *in vitro* and *in vivo* specimen. To this end, the design of automated software algorithms and correlation analysis for the detection of phases I and II biotransformation products have been developed (133–135). Nonetheless, these software packages are not meant to be a replacement for the scientific expertise and experience of a trained analyst. Thus, it is critical to have a sound understanding of ADME concepts and an appreciation of the judicious use of any automated software package. Consequently, it is recommended to perform manual inspections of the raw MS and MS/MS output to avoid possible overlooking of any key pieces of information.

Other novel technological approaches such as accurate mass measurements using quadrupole time-of-flight (Q-ToF) mass spectrometry, (136–138) rapid online metabolic screening, (139) chip-based quantitative and qualitative analysis, (140–142) and drug metabolite identification using on-line LC/NMR/MS (143,144) will continue to show to be powerful and promising additions.

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Received October 1, 2001
Accepted October 27, 2001
Manuscript 5662

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