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Selected Reaction Monitoring LC−MS Determination of Idoxifene and Its Pyrrolidinone Metabolite in Human Plasma Using Robotic High-Throughput, Sequential Sample Injection

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The generation of large numbers of samples during early drug discovery has increased the demand for rapid and selective methods of analysis. Liquid chromatography−tandem mass spectrometry (LC−MS−MS), because of its sensitivity, selectivity, and robustness, has emerged as a powerful tool in the pharmaceutical industry for many analytical needs. This work presents a high-throughput selected reaction monitoring LC−MS bioanalytical method for the determination of idoxifene, a selective estrogen receptor modulator, and its pyrrolidinone metabolite in clinical human plasma samples. The described method uses short, small-bore columns, high flow rates, and elevated HPLC column temperatures to perform LC separations of idoxifene and its metabolite within 10 s/sample. Sequential injections were accomplished with a 215/889 multiple probe liquid handler (Gilson, Inc.), which aspirates eight samples simultaneously and performs its rinse cycle parallel to sample injection, resulting in minimum lag time between injections. This high-throughput method was applied to the determination of idoxifene and its metabolite in clinical human plasma samples. Sample preparation employed liquid/liquid extraction in the 96-well format. Method validation included determination of intra- and interassay accuracy and precision values, recovery studies, autosampler stability, and freeze−thaw stability. The LOQ obtained was 10 ng/mL for idoxifene and 30 ng/mL for the metabolite. Using idoxifene-d_5 as an internal standard, idoxifene showed acceptable accuracy and precision values at QC level 1 (QC1, 15 ng/mL), level 2 (QC2, 100 ng/mL), and level 3 (QC3, 180 ng/mL) (85.0% accuracy ± 12.0% precision, 95.1 ± 4.9%, and 90.3 ± 4.7%, respectively). The pyrrolidinone metabolite also showed acceptable accuracy and precision values (using no internal standard for quantitation) at QC1 (60 ng/mL), QC2 (100 ng/mL), and QC3 (180 ng/mL) (104.9 ± 14.4%, 91.1 ± 13.0%, and 90.8 ± 12.2%, respectively). The validated method was applied to the analysis of 613 human clinical plasma samples. An average run time of 23 s/sample (∼37 min/96-well plate or over 3700 sample/day) was achieved. The successful validation presented indicates that rapid methods of analysis can efficiently and reliably contribute to the fast sample turnaround required for high sample number generating processes.

The development of combinatorial chemistry techniques[1,2] as a means to produce large numbers of potential drug candidates has resulted in the need for faster methods of analysis to more efficiently target lead compounds. Similarly, time-saving cassette dosing experiments during the preclinical drug development stage require more rapid sample analysis turnaround time to obtain pharmacokinetic information.[3,4] The early stages of drug development often produce complex mixtures of drugs and byproducts or metabolites. The use of traditional analysis techniques, namely, LC−UV or LC−fluorescence, often lack the combined sensitivity and selectivity needed to analyze complex mixtures.[5,6] Lack of selectivity often results in the application of relatively long HPLC gradients to obtain sufficient separation of compounds from interfering matrix components. The emergence of LC−MS and LC−MS−MS techniques has provided a more sensitive and selective analytical tool for the pharmaceutical industry.[7−10]

Quantification of analytes in a complex mixture can be determined through the use of selected reaction monitoring (SRM) coupled with HPLC, a highly selective and sensitive analytical combination that reduces the need for lengthy chromatographic separations. Consequently, the selectivity and robust nature of LC−MS and SRM−LC−MS have provided the opportunity for the development of high-throughput methods of analysis and is now the method of choice for bioanalysis and purity determination. To illustrate the potential for fast analyses, the

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determination of benzodiazepines in human urine was reported to provide fast SRM−LC−MS analysis capability through the use of four autosamplers coupled to one LC column (1000 samples/12 h).[11] In addition, a sample analysis time of 240 injections/hour was demonstrated for the multicomponent mixture analysis of flavones, sulfonamides, benzodiazepines, and tricyclic amines from human urine and plasma.[12] Robotically performed protein precipitation in the 96-well format and a rapid gradient were used to achieve a throughput of over 400 samples/day.[13] Direct introduction of samples for in-line sample cleanup via column switching has also been successfully used to eliminate the time-consuming steps associated with off-line sample extraction procedures.[14−17]

Recently, to demonstrate that high-throughput methods are sufficiently robust for the analysis of large quantities of samples, the determination of selective estrogen receptor modulators (SERMs) in fortified human plasma was accomplished with a throughput rate of over 2000 samples/day.[18] Our study also focuses on SERMs, a group of estrogenic compounds having antagonist activity in reproductive tissues of women (i.e., breast and endometrium) and agonist activity in the desired target tissues (i.e., bone and lipid metabolism). Because of these actions, SERMs may help lower the incidence of breast and uterine cancers while preserving bone density and decreasing the risk for heart disease[19] in postmenopausal women. Tamoxifen is a widely studied SERM that has antitumor activity[20,21] but may be associated with an increased risk of endometrial cancer.[22] For this reason, interest has risen in the study of tamoxifen derivatives for the treatment of breast cancer and other postmenopausal associated diseases with the hope of reducing side effects.[23] Idoxifene, a halogenated tamoxifen derivative, has a greater affinity for the estrogen receptor than tamoxifen and is less uterotrophic.[24,25] Idoxifene has also been shown to reduce bone loss and to lower cholesterol levels in rats, suggesting that it may be effective for treatment of osteoporosis and other vascular-related diseases associated with menopause.[26]

This paper presents the high-throughput determination of idoxifene and its pyrrolidinone metabolite in human clinical plasma samples using a validated[27,28] SRM−LC−MS method. Fast chromatography and rapid, sequential injections are used to achieve an HPLC separation of less than 10 s and a sample-to-sample cycle time of 23 s.

EXPERIMENTAL SECTION

Materials and Methods. HPLC-grade acetonitrile, methanol, dimethyl sulfoxide, and hexane were obtained from J. T. Baker (Phillipsburg, PA). Isoamyl alcohol (3-methyl-1-butanol) was from Aldrich (Milwaukee, WI). Formic acid (88%, double distilled) was from GFS Chemicals, Inc. (Columbus, OH). Deionized water was generated in-house with a Barnstead Nanopure II filtration system (Boston, MA). Human plasma was obtained from Lampire Biological Laboratories (Coopersburg, PA). Plasma used was from donors 39433, 35168, 40565, 33691, 40214, and 35449. Analytical standards of idoxifene, idoxifene-d_5, and idoxifene's pyrrolidinone metabolite were generously donated by the SmithKline Beecham Pharmaceutical Co. The 96-well plates were purchased from Phenix Research Products (Hayward, CA), and sealing Cap Mats were purchased from Matrix Technologies Corp. (Hudson, NH).

Sample Preparation. Liquid/liquid extraction was performed in 1.2-mL polypropylene 96-well plates.[29] All plasma was pipetted manually using a 200-μL Pipetteman (Gilson Inc., Middleton, WI). Pipetting of solvents and transfer of liquid/liquid extraction layers was performed robotically using a Tomtec (Hamden, CT) Quadra 96 model 320 robotic pipettor.

For liquid/liquid extraction, 100 μL of plasma was extracted with 25 μL of acetonitrile combined with 400 μL of 4% isoamyl alcohol in hexane.[18] The 96-well plates were sealed with Cap Mats, mixed for 10 min on a Fisher (Fisher Scientific, Pittsburgh, PA) hematological mixer modified to hold up to four 96-well plates, and then centrifuged for 5 min at 3800 rpm at 18 °C using an Eppendorf (Brinkmann Instrument Co., Westbury, NY) 5810R centrifuge with a four-plate rotor. The upper layers were then robotically transferred to a fresh 96-well plate via the Tomtec pipettor. The resulting organic layers were evaporated to dryness in a 65 °C water bath (Precision Scientific reciprocal shaking water bath model 25) under a gentle stream of nitrogen using an in-house-constructed blow-down apparatus.[29] The dried samples were then reconstituted in 120 μL of deionized water/mobile phase (50:50). The plates were sealed with Cap Mats, mixed on a vortex mixer for 30 s, mixed on the hematological mixer for 10 min, and then centrifuged at 3800 rpm for 5 min at 18 °C. The plates were sealed with aluminum foil (Reynolds Metals Co., Richmond, VA) and analyzed by SRM−LC−MS immediately after preparation unless otherwise indicated. Sample injection was performed through the aluminum foil seal (injection through the Cap Mats was not possible because the Gilson 215/889 multiple probe was not equipped with septum piercing needles).

Preparation of Standards and QCs. Stock solutions of idoxifene, idoxifene-d_5, and the pyrrolidinone metabolite were made in methanol at a known concentration of ~2 mg/mL. From the 2 mg/mL stock solutions were prepared 0.5 mg/mL solutions

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in 75% methanol/25% water. The 0.5 mg/mL analytical solutions were then used to generate stock solutions in human control plasma of idoxifene, idoxifene-d_5, and the pyrrolidinone metabolite at known concentrations of approximately 10 and 100 ng/μL (to avoid precipitating plasma proteins, plasma was never spiked with more than 10% methanol). The final working plasma stock solution of idoxifene-d_5 was made at a concentration of 0.16 ng/μL (generated from the 10 ng/μL plasma stock solution). All plasma stock solutions were prepared in 15-mL polypropylene conical screw-cap centrifuge tubes (VWR, South Plainfield, NJ), and were mixed on the Fisher hematological mixer for 10 min before use. Standards and QCs were prepared in human control plasma from analyte stock solutions created from separate weighings of idoxifene and the pyrrolidinone metabolite. Stock solutions for idoxifene-d_5 were made from only one weighing. Standards were prepared in 15-mL polypropylene conical centrifuge tubes at eight calibration levels in replicates of three (each replicate was made using a different plasma donor, resulting in three separate "batches" of standards). QCs were prepared in 60-mL wide-mouth polypropylene bottles at four concentration levels in replicates of three (with each replicate made with a different plasma donor). Both standards and QCs were mixed thoroughly on the hematological mixer for 10 min and were either used immediately or frozen at −20 °C until further use.

Extraction Efficiency, Signal Suppression, and Method Validation. To determine liquid/liquid extraction efficiency, 100 μL of human control plasma was spiked before extraction (preextract spike). Additionally, wells containing blank plasma were first extracted and then spiked prior to the nitrogen evaporation step (postextract spike). Spiking of 100 μL of plasma was done directly in the 96-well plate with 10 ng/μL stock solutions of the analytes. Extraction efficiency was determined at QC levels 1, 2, and 3, in replicates of eight. Extraction efficiency was calculated by comparing the SRM−LC−MS signal intensity of the postextract spike to that from the preextract spike.

To determine the electrospray ionization suppression effects of the plasma matrix on idoxifene and its metabolite, experiments were done according to the method of Bonfiglio et al.[30] Briefly, idoxifene and the pyrrolidinone metabolite (1×10^{-5} M) were infused postcolumn at 10 μL/min via a tee junction. LC conditions were as described below. In the SRM mode, ion current signal was collected for 2 min to obtain a baseline level, and then either a mobile-phase blank or a blank plasma liquid/liquid extract was injected. Ion current signal was recorded until the signal returned to baseline levels (i.e., when matrix components had eluted from the column). To ensure that no late-eluting matrix components were present, ion current was recorded for 20 min; no drop in signal was observed after the initial elution of weakly retained compounds.

For method validation studies, three batches of standards and QCs were prepared, with each batch representing human control plasma from a different donor. Seven calibration standards (10, 20, 40, 90, 100, 150, and 200 ng/mL for idoxifene and 30, 40, 80, 100, 120, 150, and 200 ng/mL for the pyrrolidinone metabolite), three QC levels, and a dilution QC (QC1 = 15 ng/mL for idoxifene and 60 ng/mL for the metabolite; QC2 = 100 ng/mL for both analytes; QC3 = 180 ng/mL for both analytes; dilution QC = 500 ng/mL for both analytes) were prepared. Each batch was prepared in a 96-well plate, extracted, and analyzed by SRM−LC−MS, with the standards located in increasing concentration sequence at the beginning and end of each plate. The internal standard was added to the samples in the plates prior to liquid/liquid extraction (50 μL of a idoxifene-d_5 0.16 ng/μL plasma stock solution was added with an Eppendorf Repeater Plus pipettor). The final concentration of idoxifene-d_5 in all human plasma batches was 52 ng/mL. The results from the first batch were used to determine the intraassay precision and accuracy, and the interassay precision and accuracy were determined using the results from all three batches.

For freeze−thaw stability studies, QC1, QC3, and standards from three batches were subjected to three freeze−thaw cycles as described by Shah et al.[27,28] On the third thaw, sample extracts were prepared and analyzed as described above. The results were compared to those obtained on the day of initial sample preparation.

Autosampler stability was determined by preparation of two plates containing standards placed in increasing order of concentration at the beginning and end of each plate, QCs at 3 levels (in replicates of 4/plate, randomly distributed throughout plate), and 68 clinical human plasma samples. The plates were extracted and analyzed by SRM−LC−MS immediately after preparation and after 8 h at room temperature (the plates were sealed with aluminum foil). A change in analyte concentration of more than 15% was considered unacceptable autosampler stability.

Reversed-Phase HPLC and Mass Spectrometry. RP-HPLC was performed on a Phenomenex (LUNA series) 1 mm × 30 mm C18 column, 3-μm particle size. The LC pump was a Hitachi (Tokyo, Japan) L-6200A intelligent pump, and the mobile phase consisted of 85% acetonitrile/14% water in 1% formic acid. The flow rate was 0.7 mL/min, and the column temperature was maintained at 70 °C (LC-22A temperature controller and column heater; Bioanalytical Systems Inc., West Lafayette, IN); the column pressure was ∼2100 psi under these conditions.

SRM−LC−MS analyses were performed on a PE Sciex (Ontario, Canada) API 3000 triple quadrapole mass spectrometer equipped with a TurboIonSpray source operated in the positive ion mode. The TurboIonSpray gas was heated to 425 °C at a flow of 7.5 L/min, and the optimized spray voltage was 2000 V. Nitrogen nebulizing, curtain, and CAD gases were set at 15, 15, and 10 (arbitrary units), respectively. The collision energy was 42 V, and the following transitions were monitored in the SRM mode: idoxifene, m/z 524 → 98; idoxifene-d_5, m/z 529 → 98, and the pyrrolidinone metabolite, m/z 538 → 112. The analytes were each monitored with a dwell time of 50 ms and a pause time of 2 ms.

The autosampler used was a Gilson Inc. 215/889 multiple probe liquid handler equipped with 10-μL loops and a Rheodyne (Cotati, CA) LabPro series 10-port selector valve. The 889 eight-valve injection module incorporates six-port two-position injection valves whose loops are loaded in parallel through eight injection ports. The selector valve controls each injection by directing the total HPLC flow through each sample loop in turn. The needles and the ports are rinsed normally under control of the sampler software while the injections take place. The selector valve allowed the 215/889 to complete its rinse protocol and to aspirate the next

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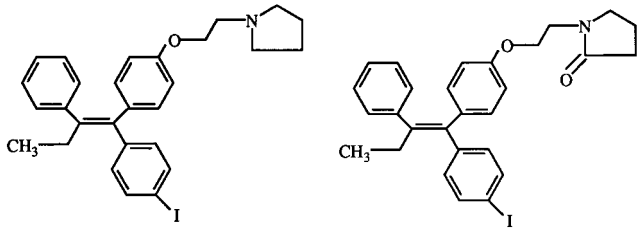

Figure 1. Structures of idoxifene and its pyrrolidinone metabolite.

set of eight samples parallel to sample analysis by SRM−LC−MS. The injector rinse solvent was methanol/acetonitrile/dimethyl sulfoxide/water (35:35:20:10). Full loop injections were performed under the following conditions: push volume (volume required to fill tubing leading from the injection port to the injection valves), 15 μL; sample aspiration volume, 30 μL; air gap volume, 15 μL. To reduce carryover, 200 μL of the rinse solvent was aspirated following sample and air gap aspiration. This allowed the eight Rheodyne injector valves to be rinsed while in the load position. Communication between the 215/889 and the mass spectrometer was accomplished via contact closures.

Data were acquired for each LC run for 13 s; this accommodated any shifts in retention time (shifts of approximately 0.5−1 s was observed for all analytes depending on the injector probe used for injection). The mass spectrometer data system needed approximately 3 s to download parameters and prepare for the next acquisition. For successful contact closure communication, a pause of 4 s was needed after the mass spectrometer ready signal, following which the selector valve switched to the next sample. Finally, ∼10 s was needed after every set of eight samples for the Multiple Probe to aspirate rinse solvent and load injection loops. These "overhead" times contributed to an approximate sample-to-sample cycle time of 23 s.

Safety. All plasma purchased contained a certificate of testing stating that all plasma had been tested for hepatitis B surface antigen, anti HIV-1/HIV-2, syphilis, anti-hepatitis C, and HIV-1 antigens. However, all samples were treated as potential biohazards and were handled with the proper protective clothing, gloves, eye wear, and laboratory precautions.

RESULTS AND DISCUSSION

The structures of the SERM idoxifene and its pyrrolidinone metabolite are shown in Figure 1. The Q1 and product ion mass spectra for idoxifene and its metabolite are shown in Figures 2 and 3, respectively.

Sample workup involved liquid/liquid extraction in the 96-well format of human plasma using 4% isoamyl alcohol in hexane. This extraction resulted in clean extracts that permitted analysis of almost 1500 plasma extracts with no increase in column pressure and no clogging of tubing or frits, as described in previous work.[18] The percentage recovery of idoxifene and the pyrrolidinone metabolite was determined for QC1, QC2, and QC3 (Table 1). The recovery of idoxifene at QC1 was ∼20% lower than QC2 and QC3, which was probably a reflection of adsorption effects of the drug to the surfaces of the plate.

The presence of coextracted matrix components may cause signal suppression of certain analytes. The degree of signal suppression was determined by postcolumn infusion of idoxifene and the pyrrolidinone metabolite.[30] The mobile-phase conditions were as described earlier under the Experimental Section. Ion current signal (in SRM mode) was collected to provide a representative signal from known levels of the target analytes infused postcolumn while the injection of a liquid/liquid extract human control plasma blank was made. The reduction in ion current signal following injection of the plasma blank provided an indication of the degree of signal suppression on the targeted test articles caused by extracted matrix components. Very little signal suppression was observed with idoxifene. At higher voltages (4200 V), more signal suppression was observed, possibly because higher voltages ionize more matrix components. Additionally, at high flow rates (0.7 mL/min), ramping of the ion spray voltage produced a Gaussian-like ion current response from idoxifene,

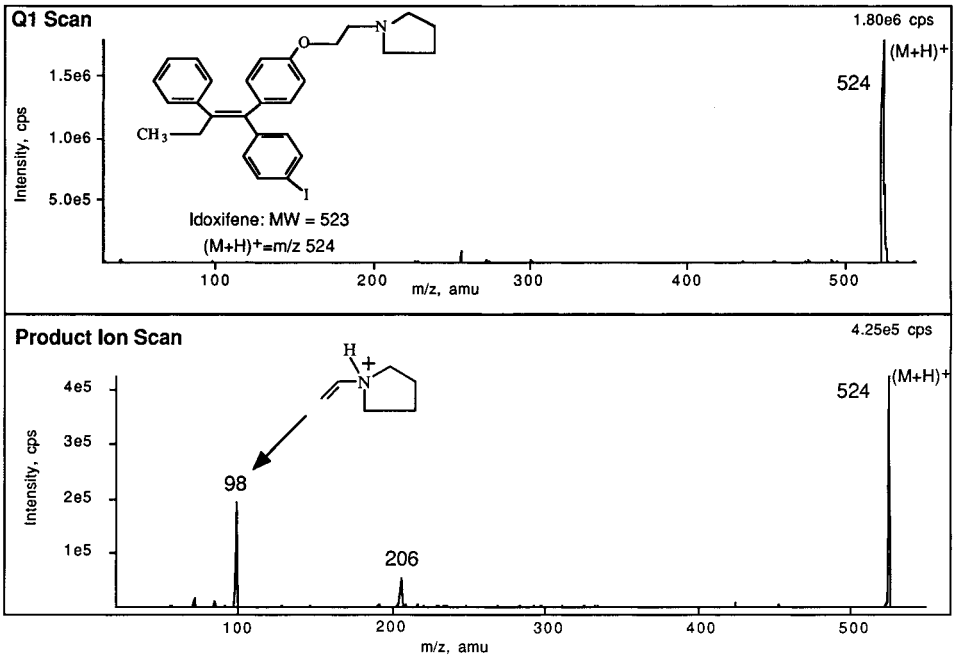

Figure 2. Q1 full scan and product ion mass spectra of idoxifene.

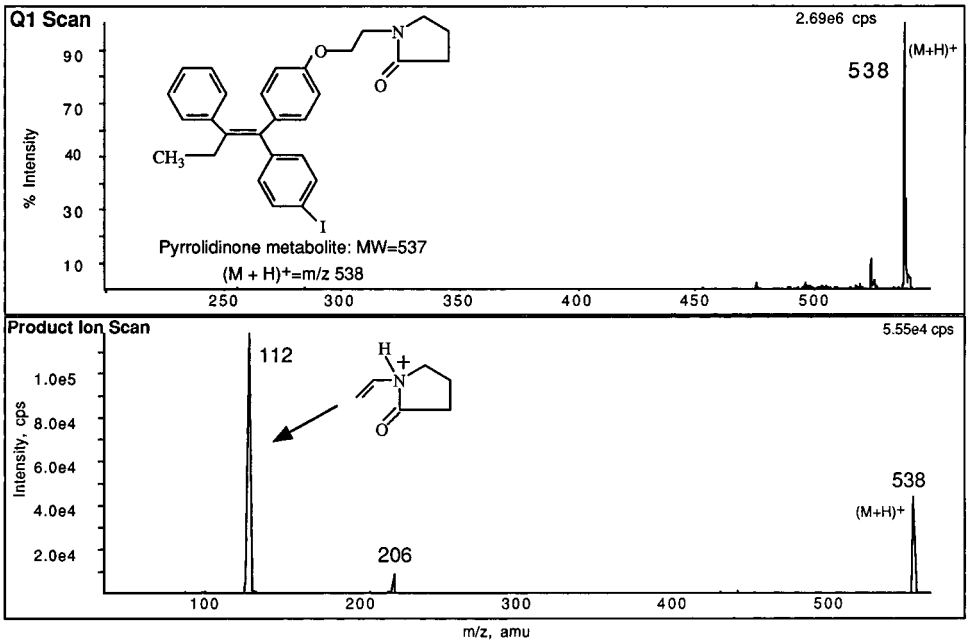

Figure 3. Q1 full scan and product ion mass spectra of the pyrrolidinone metabolite.

Table 1. Liquid/Liquid Extraction Recoveries from Spiked Human Control Plasma

	% recovery (±CV)	
QC	idoxifene	pyrrolidinone metabolite
QC1[a]	44 ± 11	56 ± 11
QC2[b]	66 ± 9	63 ± 10
QC3[c]	68 ±9	56 ±6

[a] QC1 = 15 ng/mL for idoxifene and 60 ng/mL for the metabolite. [b] QC2 = 100 ng/mL for both analytes. [c] QC3 = 180 ng/mL for both analytes.

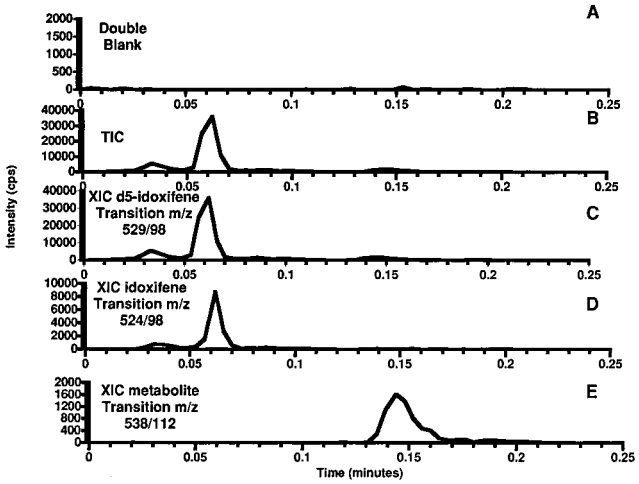

Figure 4. SRM−LC−MS chromatogram from a human control plasma liquid/liquid extract blank and an extract spiked at LOQ (10 ng/mL for idoxifene and 30 ng/mL for the metabolite). (A) Double blank; (B) TIC; (C) XIC of idoxifene-d_5 (transition m/z 529 → 98); (D) XIC of idoxifene (transition m/z 524 → 98) (E) XIC of metabolite (transition m/z 538 → 112). SRM−LC−MS conditions were as follows: mobile phase, 85% acetonitrile/15% water in 1% formic acid; flow, 0.7 mL/min; column, 1 mm × 30 mm C18, 3-μm particle size (Phenomenex, LUNA series); column temperature, 70 °C; 10-μL full loop injection; detection, SRM−LC−MS.

with the maximum signal occurring at ~2000 V. Thus, use of a lower ion spray voltage reduced signal suppression effects by both increasing the ionization efficiency of idoxifene and possibly reducing the amount of matrix components ionized. This low-voltage phenomenon was not observed for the metabolite (the ion current signal remained relatively stable over a range of ion spray voltages). Approximately 40% of the pyrrolidinone metabolite's ion current signal was suppressed (at its retention time) upon injection of a blank plasma extract. This signal suppression, combined with lower ionization efficiency compared to idoxifene, resulted in higher limits of quantitation (LOQ) for the pyrrolidinone metabolite.

The lowest values in the standard curve (LOQ) for the determination of idoxifene and the pyrrolidinone metabolite in human plasma were 10 and 30 ng/mL, respectively. The SRM chromatogram and extracted ion chromatogram (XIC) of a human control plasma extract spiked at the LOQ is shown in Figure 4. The small peak eluting prior to idoxifene was also observed with idoxifene-d_5. For both idoxifene and idoxifene-d_5, the product ion mass spectrum of the smaller peak was identical to that from the larger peak. The possibility of peak-splitting is not likely because the reconstitution solvent was weaker than the mobile phase. Furthermore, the two peaks are observed regardless of the injection solvent strength. Purity results for idoxifene-d_5 indicated the presence of the *Z*-isomer. We therefore believe that the small peak seen with both idoxifene and idoxifene-d_5 represents small amounts of the *Z*-isomer, while the large peak represents the *E*-isomer. Though not visible using the LC conditions in Figure 4, the pyrrolidinone metabolite also exhibits two peaks if the chromatography conditions are altered to provide improved separation.

Idoxifene was quantitated on the basis of its area ratio relative to the area of the internal standard, idoxifene-d_5. The pyrrolidinone metabolite, however, was better quantitated without the internal standard, suggesting that the deuterated parent drug was not a suitable internal standard for the metabolite. For both idoxifene

Table 2. Intraassay Accuracy and Precision Results from Method Validation Experiments

	accuracy (% target) ± CV (n = 10)	
QC	idoxifene	pyrrolidinone metabolite
QC1[a]	85.0 ± 12.0	104.9 ± 14.4
QC2[b]	95.1 ± 4.9	91.1 ± 13.0
QC3[c]	90.3 ± 4.7	90.8 ± 12.2
dilution QC[d]	110.9 ± 12.6	95.7 ± 18.8

[a] QC1 = 15 ng/mL for idoxifene and 60 ng/mL for the metabolite. [b] QC2 = 100 ng/mL for both analytes. [c] QC3 = 180 ng/mL for both analytes. [d] Dilution QC = 500 ng/mL (diluted 10-fold) for both analytes.

Table 3. Interassay Accuracy and Precision Results from Method Validation Experiments

	accuracy (% target) ± CV (n = 30)	
QC	idoxifene	pyrrolidinone metabolite
QC1[a]	86.9 ± 8.5	104.1 ± 14.4
QC2[b]	93.0 ± 9.7	96.7 ± 14.5
QC3[c]	90.2 ± 8.3	91.4 ± 13.1
dilution QC[d]	117.1 ± 14.8	99.7 ± 20.6

[a] QC1 = 15 ng/mL for idoxifene and 60 ng/mL for the metabolite. [b] QC2 = 100 ng/mL for both analytes. [c] QC3 = 180 ng/mL for both analytes. [d] Dilution QC = 500 ng/mL (diluted 10-fold) for both analytes.

and the pyrrolidinone metabolite, intraassay accuracy and precision values met acceptance criteria at QC1, QC2, and QC3. Accuracy and precision values for idoxifene at the dilution QC met normal acceptance criteria, while the precision values for the pyrrolidinone metabolite did not. In general, precision values for the metabolite tended to vary more widely than those for the parent drug. Had we possessed a deuterated analogue of the metabolite, it is likely that this variation would have been less. The interassay validation results were similar to the intraassay results (Tables 2 and 3). Both accuracy and precision values were within acceptance criteria at QC1, QC2, and QC3, though neither analyte met criteria at the dilution QC. Because the QC dilution was performed in the wells of the plate, the mostly likely explanation for failure of the dilution QC is improper mixing prior to the extraction step.

Freeze−thaw stability was determined by performing three freeze−thaw cycles on QC1 and QC3 from three spiked human plasma batches. The results obtained following the third thaw cycle were compared with the results from the fresh sample preparation. The results from the freeze−thaw experiments verified that both idoxifene and the pyrrolidinone metabolite were stable (the analyte lost did not exceed 10%) through three freeze−thaw cycles (data not shown).

Two plates were prepared that contained 68 representative clinical human plasma samples, QCs 1−3 (in replicates of 4) and standard curves at the beginning and end of each plate in ascending order of concentration. The two plates were analyzed immediately after preparation and again after remaining at room temperature (sealed with aluminum foil) for 8 h. A comparison between the 0- and 8-h results confirmed that the samples were stable (analyte loss did not exceed 10%) at room temperature on the autosampler tray (autosampler stability, data not shown). This experiment also provided a representative selection of clinical plasma samples from which the relative concentration range of drug and metabolite could be determined. These initial runs showed that idoxifene was present in the clinical samples at levels between QC1 and QC2, while the metabolite was present closer to the LOQ. None of the 68 clinical samples analyzed contained idoxifene or the metabolite at a level outside the range of the standard curve, which suggested dilution of clinical samples would not be necessary.

For complete analysis of all 613 clinical human plasma samples, 16 96-well plates were needed. Each plate contained standard curves at the beginning and end in ascending order of concentration, QCs 1−3 dispersed randomly (in replicates of 3), 4 double blanks, and 40 clinical samples. The clinical samples were manually pipetted into the plates. Because of the time-consuming process of manually pipetting 613 clinical samples from individual test tubes, the 16 prepared plates were sealed with Cap Mats following extraction of the samples and frozen at −20 °C overnight. It is suggested that future sample collection strategies could incorporate collecting the individual patient samples directly in the 96-well format, which would preclude the manual pipetting step. The next morning, the plates containing the frozen sample extracts were thawed, mixed thoroughly on a vortex mixer, centrifuged at 3800 rpm at 18 °C for 5 min, and then analyzed by SRM−LC−MS. Total analysis time was 9 h and 37 min (∼37 min/plate, ∼23 s/sample). LC column pressure remained stable throughout the entire analysis.

An approximate 45% signal loss was observed over the course of the analytical run. However, the standard curves retained their quality throughout the analysis, with the area ratios of idoxifene/idoxifene-d_5 remaining constant throughout plates 1−16 (the difference in area ratios at each standard curve value between plate 1 and plate 16 did not exceed 15%). Additionally, the slope and correlation coefficient from plate 1 was 0.024 32 and 0.990 84, respectively, and 0.027 84 and 0.993 49 from plate 16. Furthermore, completion of data analysis indicated that accuracy and precision values of the QCs from all plates remained within acceptance criteria. Retention times also remained stable, as shown in Figure 5, which compares the retention time of clinical sample 75 (plate 2) and clinical sample 607 (plate 16). Thus, despite the loss in signal, reliable quantitative results were obtained.

Of the 613 clinical samples analyzed, idoxifene was detected in 65% (of these, 8% were less than or equal to the LOQ). The pyrrolidinone metabolite was detected in only 23% of the samples, and of these, 67% were less than or equal to the LOQ. This indicated that a lower LOQ for the metabolite was needed to reliably quantitate the metabolite in the given clinical samples. It should be noted that the signal loss observed during the run was not completely responsible for the detection difficulties of the metabolite. Even in the early stages of the analysis, levels of the metabolite in the clinical samples were too low for reliable quantitation. Unfortunately, very fast analysis such as those described here usually affect an elevated LOQ for the method. Consequently, these high-throughput SRM−LC−MS strategies are best suited for those applications where less stringent method validation criteria are acceptable and where low detection limits can be sacrificed for the time-saving benefits of high-throughput screening methods.

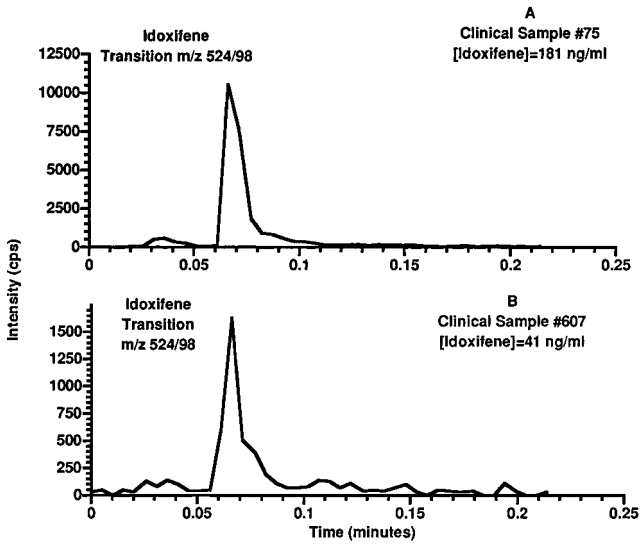

Figure 5. SRM−LC−MS ion chromatograms of idoxifene from clinical human plasma samples 75 (A) and 607 (B) illustrating retention time stability over the course of 9 h. The clinical samples (100 μL) were extracted as described under Experimental Section. SRM−LC−MS conditions are as described in Figure 4.

CONCLUSIONS

We have presented a validated method for the determination of a parent drug and its metabolite in clinical human plasma samples using SRM−LC−MS and rapid injection strategies that further push the limits of high-throughput bioanalytical analyses. Liquid/liquid extraction of human plasma using 96-well plates produced clean extracts that allowed continuous analysis of ∼1500 plasma extracts (including clinical samples, standards, and QCs) with no significant increase in column pressure. Though a decline in SRM−LC−MS ion current signal was observed over the course of the run, the quality of the standard curves and retention times remained acceptable. The LOQ for the pyrrolidinone metabolite was too high for successful quantitation in clinical samples, illustrating the need for further research into methods of detection that increase detection limits of weakly ionized neutral compounds. However, though LOQs were somewhat high, they should be applicable to areas such as drug discovery and combinatorial chemistry analysis where sensitivity is not necessarily a concern. We also accomplished the successful validation of a metabolite without the use of an internal standard; because internal standards are not always available during drug discovery studies, this capability is relevant to drug discovery studies.

Despite the relatively fast sample turnaround presented here, some bottlenecks still need to be addressed. For example, the extensive amount of time needed to manually pipet hundreds of clinical samples would be eliminated by collecting the samples directly into 96-well plates. Also, data analysis needs to be fully integrated and automated in order to minimize the time needed to prepare the data for presentation.

The high-throughput conditions presented proved to be sufficiently robust for the analysis of more than 1000 samples (including QCs and standards) and also produced quality results that met current method validation acceptance criteria. Although a full day was not required to analyze the described set of 613 clinical plasma samples, the validated high-throughput bioanalytical method used operated at a rate sufficient to analyze ∼3900 samples in 24 h. Such rapid sample turnaround would greatly benefit studies in which the rapid analysis of large numbers of samples results in more efficient and more cost-effective methods of drug discovery or sample screening.

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