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Quantitative Determination of Orlistat (Tetrahydrolipostatin, Ro 18-0647) in Human Plasma by High-performance Liquid Chromatography Coupled with Ion Spray Tandem Mass Spectrometry

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A rapid, sensitive and specific analytical method was developed and validated to quantify tetrahydrolipostatin (Orlistat, Ro 18-0647) in human plasma in order to provide pharmacokinetic data from clinical trials. This method employs a preliminary plasma protein precipitation step followed by a simple, one-step liquid–liquid extraction procedure to isolate Ro 18-0647 and its pentadeuterated internal standard, Ro 18-0647-d_5, from the biological matrix. Reconstituted extracts were analyzed by liquid chromatography/ion spray tandem mass spectrometry (LC/MS/MS) in the selected reaction monitoring (SRM) mode. Chromatography was carried out using a 2 mm i.d. × 50 mm Deltabond Phenyl column. The eluent was acetonitrile–2 mM ammonium acetate (90:10). The retention time of the analyte was 1.2 min and chromatographic run times were less than 1.5 min. No interferences from anticoagulants, collection devices or endogenous constituents of the plasma were observed. The assay has a lower limit of quantitation (LLQ) of 0.20 ng ml^{-1} plasma and a lower limit of detection (LLD) of 0.10 ng ml^{-1} plasma, based on 1 ml aliquots. The capability to detect 0.025 ng ml^{-1} in plasma has also been demonstrated. The calibration graphs were linear from 0.20 to 10 ng ml^{-1}. The assay was initially validated with a linear range of 0.20–1.0 ng ml^{-1}. This range was later extended and validated to an upper level of quantitation of 10 ng ml^{-1}. Intra- and inter-assay precision studies showed a mean variability of less than 10%. The recovery, inter-assay precision and accuracy of the method were within acceptable bioanalytical standards. The assay has been shown to reliably provide automated, unattended sample analysis for ~150 samples per day. In an additional series of tests, Ro 18-0467 was shown to be stable under conditions that might be encountered during the analysis of samples from clinical trials. This LC/MS/MS assay procedure for Ro 18-0647 in human plasma has proven to be robust, sensitive, specific, accurate and reproducible. This method has been used to analyze over 5000 study samples.

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INTRODUCTION

Tetrahydrolipostatin (THL) (Ro 18-0647) is a specific lipase inhibitor derived from lipostatin, a lipid produced by *Streptomyces toxytrincini*. It is an inhibitor of gastric, carboxyl ester and pancreatic lipases and it specifically reduces the absorption of dietary fat due to the inhibition of triglyceride hydrolysis.[1] THL is currently under clinical evaluation for the treatment of obesity. Its chemical synthesis has also been reported[2] and its chemical structure is shown in Fig. 1(A). THL has been prepared by the hydrogenation of lipostatin following isolation of the latter from a strain of *Streptomyces toxytricini* a.[3] These authors also prepared both compounds by total synthesis. In addition to pancreatic lipases, it has been shown that Ro 18-0647 also inhibits human gastric lipase, carboxyl ester lipase (cholesterolesterase) of pancreatic origin, and the closely related bile salt-stimulated lipase from *Rhizopus arrhizus* or lipase recently isolated from *Staphylococcus aureus*.

THL acts locally in the gastrointestinal (GI) tract and yields low, if not undetectable, plasma concentrations when administered orally. Efficacy and toxicological effects have been reported to be favorable with no significant carry-over effects.[4] Its thermal and hydrolytic degradation have been reported, and all main degradation products isolated, characterized and synthesized.[5] The THL degradation products produced by human carboxyl ester lipase have also been described.[6]

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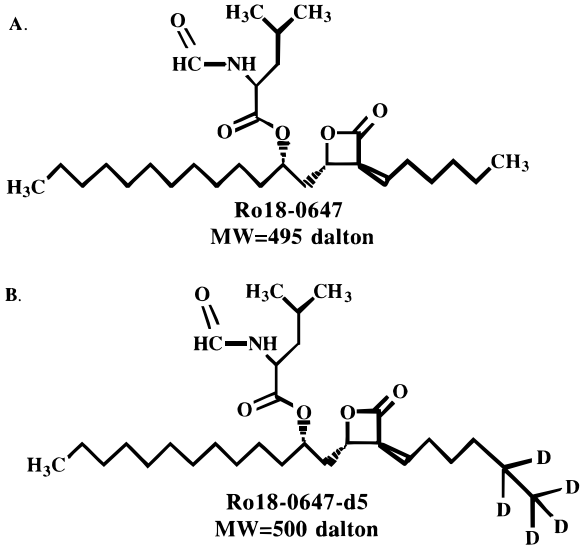


Figure 1. Structures of the parent drug and its pentadeuterated internal standard.

Samples from human and animal (rat, dog and mouse) clinical studies have been analyzed previously for Ro 18-0647 by positive ion ammonia chemical ionization (CI) via gas chromatography/mass spectrometry (GC/MS) and gas chromatography/tandem mass spectrometry (GC/MS/MS).[7] The GC/MS/MS method depended upon the quantitative thermal elimination of carbon dioxide from the lactone ring of the parent compound in the hot injection port of the gas chromatograph. The low-level quantitative results obtained from this analytical approach displayed considerable variability. In addition, the lower limit of quantitation (LLQ) obtained from GC/MS/MS was only 1–5 ng ml^{-1} using 1 ml of plasma.[7] Since many of the clinical samples showed no detectable quantities for Ro 18-0647 using this GC/MS/MS approach, there was concern that a more sensitive assay was needed to quantify Ro 18-0647 reliably at sub-nanogram concentrations in plasma.

We report here an improved quantitative method based on liquid chromatography coupled with ion spray tandem mass spectrometry[8–10] (LC/MS/MS). LC/MS/MS was chosen over UV detection owing to the poor sensitivity and specificity of the latter for this compound. Because THL is thermally labile,[5] the compound was predicted to be a good candidate for the LC ion spray MS/MS approach since ionspray involves ambient temperature and very mild ionization experimental conditions. The objective was to establish an LLQ of 0.20 ng ml^{-1} for routine analysis and to validate a simple extraction procedure combined with a short chromatographic analysis time, which would provide rapid, selective, accurate, reproducible and reliable quantitative results by LC/MS/MS. A pentadeuterated (d_5) internal standard [Fig. 1(B)] was selected to provide the most reliable and accurate quantitative results. The use of a pentadeuterated internal standard also avoids any interference from naturally occurring carbon, hydrogen or oxygen isotopes. The LC/MS/MS method described here provides a significant increase in sensitivity, selectivity and sample throughput over previously described methods.

EXPERIMENTAL

Chemicals

Ro 18-0647 and its pentadeuterated analogue Ro 18-0647-d_5 (Fig. 1) were obtained from Hoffmann-La Roche (Nutley, NJ, USA). Their purity was determined by the Hoffmann-La Roche Quality Assurance Department to be 99.6% and 97.6%, respectively. This purity was verified for both compounds by LC/ionspray MS). The atom enrichment for the Ro 18-0647-d_5 was determined to be 99.95% by LC/ionspray MS experiments performed at unit mass resolution (0.5 Da at half-height, 10% valley). All solvents were obtained from Burdick & Jackson through Baxter/Scientific Products (McGaw Park, IL, USA). Ammonium acetate was obtained from J. T. Baker (Danvers, MA, USA). Purified distilled water (18 MΩ) was either purchased from Baxter/Scientific Products or obtained from a NANOpure-UV/B pure water purification system (Barnstead, Dubuque, IA, USA). Control human plasma (potassium oxalate) was obtained from Biological Specialty (Lansdale, PA, USA).

Instrumentation and chromatographic conditions

All method development was done on-line using high-performance liquid chromatography (HPLC) coupled with an ionspray tandem mass spectrometer system. The HPLC system used was a Hewlett-Packard 1090M equipped with an autoinjector (Hewlett-Packard, Avondale, PA, USA). The HPLC column used for the validation was a 2 mm i.d. × 50 mm Deltabond Phenyl (5 μm particles) obtained from Keystone Scientific (Bellefonte, PA, USA). Samples were reconstituted in 30 μl of acetonitrile–2 mM ammonium acetate (70:30), mixed on a vortex mixer and transferred into injection vials. The injection volume was 10 μl. The eluent was acetonitrile–2 mM ammonium acetate (90:10) maintained at a flow rate of 200 μl min^{-1} with the total HPLC effluent directed to the ionspray interface of the LC/MS system. These conditions produced a retention time of 1.2 min and a chromatographic run time of less than 1.5 min. The column and autoinjector were maintained at ambient temperature.

The mass spectrometer used was a Perkin-Elmer (PE) SCIEX (Concord, Ontario, Canada) API III atmospheric pressure ionization tandem triple-quadrupole system equipped with an articulated ionspray interface. The instrument was operated in the positive ion mode under LC/ionspray MS conditions. The declustering potential was 38 V. The sprayer and multiplier voltages were +4600 and −4200 V, respectively. The curtain gas was ultra-high-purity nitrogen maintained at a flow rate of 1.2 l min^{-1}. The nebulizing gas was delivered from a 180 l liquid nitrogen Dewar vessel at 60 psi. The instrument was operated in the MS/MS mode and data were acquired under selected reaction monitoring (SRM) conditions. The following precursor-to-product ion reactions were monitored with dwell times of 350 ms

each: m/z 496–160 and m/z 501–160 for Ro 18-0647 and the internal standard, respectively. Argon was used as the collision gas at a thickness of 500×10^{12} atoms cm^{-2}. Mass spectrometer optimization and mass axis calibration were initially performed by infusion of an equimolar solution of polypropylene glycols (PPGs) of average relative molecular mass 425 and 1000 at 10 pmol μl^{-1} in 50% aqueous methanol. This was followed by final tuning and mass axis accuracy verification by infusion of an authentic standard of Ro 18-0647 dissolved in acetonitrile–2mM ammonium acetate (70:30). The mass peak widths at half-height under these conditions was 0.6–0.7 Da for the precursor ions in the first quadrupole (Q1) and 0.6–0.7 Da in the third quadrupole for the product ions monitored in Q3.

The data were collected using the PE SCIEX proprietary software program RAD, version 2.2. Peak areas were integrated and linear regression performed by the PE SCIEX proprietary software program MacQuan, version 1.1.2.

Preparation of Standards and samples

Stock standard solutions of Ro 18-0647 and its internal standard, Ro 18-0647-d_5, were prepared as 100 $\mu g\ ml^{-1}$ solutions in methanol. The methanolic Ro 18-0647 stock standard solution was used to spike blank, drug-free control plasma to provide a 1 ng ml^{-1} plasma spiking solution. Calibration standards were prepared in duplicate from this plasma spiking solution for each analytical run. Serial dilutions of the stock plasma spiking solution provided concentrations of the plasma calibration standards for the initial validation experiments at levels of 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 ng ml^{-1}.

Quality control (QC) samples were prepared by a second individual at low, medium and high level concentrations of 0.248, 0.496 and 0.868 ng ml^{-1} from a separate 100 $\mu g\ ml^{-1}$ Ro 18-0647 solution. The 100 $\mu g\ ml^{-1}$ methanolic Ro 18-0647-d_5 stock solution was diluted to provide a 10 ng ml^{-1} spiking solution. A 50 μl volume of the 10 ng ml^{-1} internal standard solution was added to all standards, QCs and study samples, yielding a concentration of 0.50 ng ml^{-1}.

Subsequent experiments were performed to extend the range of the calibration graph to 10 ng ml^{-1}. The 100 $\mu g\ ml^{-1}$ methanolic Ro 18-0647 stock solution was used to spike blank control plasma to provide a 10 ng ml^{-1} plasma spiking solution. This plasma solution was then used to prepare duplicate calibration standards at concentrations of 0.20, 0.30, 0.40, 0.50, 0.75, 1, 5 and 10 ng ml^{-1}. Quality control samples were prepared at concentrations of 0.35, 4.32 and 8.61 ng ml^{-1} for this concentration range. The 100 $\mu g\ ml^{-1}$ methanolic Ro 18-0647-d_5 stock solution was diluted in methanol to provide a 100 ng ml^{-1} spiking solution. Volumes of 50 μl of the 100 ng ml^{-1} internal standard solution were added to all calibration standards and QCs, yielding a concentration of 5 ng ml^{-1}.

On each day of analysis, duplicate 1 ml aliquots of the calibration samples, QC samples and drug-free control samples (blanks) were prepared. Experimental samples from study subjects were also pipetted as 1 ml aliquots. A single, one-step extraction with hexane was used to isolate Ro 18-0647 and Ro 18-0647-d_5 from plasma following protein precipitation with acetonitrile. Aliquots of 1 ml of plasma were placed in 13 mm × 100 mm polypropylene tubes (Elkay Products, Shrewsbury, MA, USA), and plasma proteins were precipitated with 1 ml of acetonitrile. The tubes were mixed on a vortex mixer and centrifuged for 5 min at a relative centrifugal force (RCF) of 834. The upper layer was transferred to new 13 mm × 100 mm polypropylene tubes and 5 ml of hexane was added. The tubes were rotated on a fixed-speed hematological mixer (Fisher Scientific, Rochester, NY, USA) for 20 min and centrifuged for 5 min at an RCF of 834 and the organic layer was transferred to new 13 mm × 100 mm polypropylene tubes. The tubes were placed in a Turbo Vap LV (Zymark, Hopkinton, MA, USA) and the hexane was evaporated to dryness under nitrogen at 25 °C. The residue was dissolved in 30 μl of acetonitrile–2 mM ammonium acetate (70:30).

Recovery

The recovery of Ro 18-0647 extracted from plasma was determined by comparing the SRM LC/MS peak area ratios of samples spiked with Ro 18-0647 prior to extraction (pre-extract) with the SRM LC/MS peak area ratios from blank plasma extracts spiked with Ro 18-0647 (post-extract). The internal standard was added to all samples post-extraction at a level of 0.5 ng ml^{-1}. This was performed at 0.20, 0.60 and 1 ng ml^{-1} of Ro 18-0647 and analyzed in triplicate. The overall mean recovery from the three replicates, the standard deviation and the relative standard deviations (RSD) were calculated.

Sample analysis and calculations

All calculations were based on chromatographic peak area ratios for the SRM LC/MS precursor–product ion transitions for Ro 18-0647 and the internal standard Ro 18-0647-d_5. Concentrations of Ro 18-0647 in experimental and QC samples were determined using an equation established from a linear regression of the peak area ratios *vs.* concentration data from the calibration standards. The weighting factor used in the regression was $1/x$, where x is the concentration.

Assay validation

The validation of this method consisted of intra- and inter-assay variability, lower level of quantitation, recovery and stability experiments. The intra- and inter-assay variability experiments involved the analysis of calibration standards and QC samples on three different days. The inter-assay variability was determined for the calibration standards using the mean of the duplicate daily determinations. The mean value and RSD were calculated for each standard concentration level over the three assays. For the initial studies involving the ULQ of 1 ng ml^{-1} the inter-assay precision was also

assessed from QC samples spiked with Ro 18-0647 at low, medium and high concentrations (0.248, 0.496 and 0.868 ng ml^{-1}) in plasma. This initial validated range from 0.2 to 1 ng ml^{-1} was performed based upon historical plasma PK concentrations. However, once validation was completed with this range, it was decided to extend the range to avoid reanalysis if higher plasma PK concentrations resulted from higher dosages during the drug development process. Therefore, an intra-assay cross-validation was performed to demonstrate the ability to extend the ULQ to 10 ng ml^{-1}. For the later studies where the ULQ was raised to 10 ng ml^{-1} the same validation protocol was followed, but the QC samples had levels of 0.35, 4.32 and 8.63 ng ml^{-1}. These experiments were performed in the same manner as the calibration standards above. The mean and RSD were calculated from pentuplicate measurements at each QC concentration from these separate determinations. These data were then used to calculate the intra- and inter-assay precision (RSD) by using a one-way analysis of variance (ANOVA). The intra-assay precision is represented by the within-run (within treatment) variability and the inter-assay precision by the between-run (between treatment) variability. Acceptable precision here was considered to be an RSD of $\leqslant 15\%$. The overall precision (RSD) was also calculated using the grand mean of the calculated concentrations.

Accuracy was assessed by determining the percentage error in the analysis of QC samples. The theoretical concentration of each QC sample was subtracted from the mean concentration determined from the three days of analyses. The result was divided by the theoretical value and converted to a percentage. Acceptable accuracy was defined as a percentage error (DEV) within 20% for each QC sample and an overall mean percentage error of $\leqslant 10\%$ or less. Specificity, or the existence of potential interference in the chromatograms from the biological samples or the collection device, was monitored by running control blank samples in each calibration. The absence of any chromatographic components at the same retention time as THL suggested that no chemical interferences were occurring.

Stability of Ro 18-0647 and Ro 18-0647-d_5

The stability of Ro 18-0647 in plasma was evaluated at room temperature, after storage in a freezer ($-20\,^{\circ}C$ and $-70\,^{\circ}C$), after cycles of freezing and thawing, as dry extracts and after reconstitution in the HPLC autosampler. For these experiments, the compound was spiked into drug-free plasma at the intermediate QC level (0.496 ng ml^{-1}). For each experiment, replicates were stored under the appropriate conditions (or frozen, thawed and refrozen) for different periods of time and then all analyzed simultaneously.

The stability of the stock and working solutions of Ro 18-0647 and Ro 18-0647-d_5 was determined by monitoring the chromatographic ion current ratios produced from the calibration curves. No changes in peak area ratios were observed in these samples. Full-scan acquisition of the internal standard, Ro 18-0647-d_5, indicated $>99\%$ d_5 incorporation in this compound.

RESULTS

Sensitivity and specificity

To determine the lower level of quantitation (LLQ) for the method, control plasma samples were obtained from six different individuals. Three 1 ml aliquots were taken from each of these individuals and divided into one of the following three treatments groups and assayed: (i) no Ro 18-0647 or Ro 18-0647-d_5 added (plasma blanks); (ii) spiked to 0.10 ng ml^{-1} of Ro 18-0647; or (iii) spiked to 0.20 ng ml^{-1} of Ro 18-0647. From the analysis of these samples the LLQ was determined to be 0.20 ng ml^{-1}. The RSD and DEV for this concentration level were within 9%. Although 0.10 ng ml^{-1} could be routinely detected, samples spiked with 0.10 ng ml^{-1} did not produce sufficiently accurate or precise results. The overall RSD for this concentration level was 23.83% and the %DEV for the individual samples ranged from 17.67 to -30.73%. There were no matrix or chemical interferences observed in control plasma sample extracts obtained from the six different individuals.

The assay has proven to be highly specific allowing short retention times and high sample throughput. There were no interferences observed in any control plasma blanks analyzed. The specificity of the assay procedure with regard to four known metabolites of Ro 18-0647 has been demonstrated (unpublished results). Although one might consider carrying out this assay under flow injection analysis (FIA) conditions, e.g. without the use of an HPLC column, we believe that the use of some limited chromatographic separation provides a more robust and dependable bioanalytical method.

Recovery and linearity

Recovery studies for Ro 18-0647 were carried out at three different concentrations representative of the low, intermediate and high QC levels. The overall mean recovery for Ro 18-0647 was 67% with an RSD of 6%. The recovery of the internal standard, Ro 18-0647-d_5, was not investigated because it is a deuterated analogue of Ro 18-0647 and its recovery was assumed to be equivalent to that of the parent drug. The recovery was determined to be independent of concentration of the drug in plasma.

The linear regression of the calibration graphs was fitted by a $1/x$ weighting. Coefficients of determination (R^2) ranged from 0.993 to 0.999 for the 0.20 to 1 ng ml^{-1} calibration graphs. The linear range was extended to 10 ng ml^{-1} for one run. The R^2 for this run was 0.999. The slopes for the two calibration graphs with ULQs of 1 and 10 ng ml^{-1} were 0.002 and 0.0002, respectively.

Precision and accuracy

The individual results of QC sample evaluation during validation runs are summarized in Table 1. These data

Table 1. QC summary for intra- and inter-assay validation runs[a]

Nominal concentration (pg/ml)	Parameter	Run 1	Run 2	Run 3	Grand mean
248	Mean	237	233	233	235
	RSD%	6.46	6.74	8.32	6.82
	DEV%	−4.36	−6.00	−5.71	−5.36
496	Mean	470	447	466	461
	RSD%	6.57	6.35	9.22	7.40
	DEV%	−5.21	−9.84	−6.11	−7.05
868	Mean	885	856	815	850
	RSD%	14.2	7.59	7.00	9.91
	DEV%	1.91	−1.35	−6.11	−2.07

[a] Number of measurements = 5.

Table 2. Intra- and inter-assay precision and inter-assay accuracy summary

	Nominal concentration (pg ml^{-1})		
Parameter	248	496	868
Grand mean calc. concentration (pg ml^{-1})	235	461	850
Deviation (%)	−5.36	−7.05	−2.07
Inter-assay precision (RSD, %)	2.80	1.56	0.82
Intra-assay precision (RSD, %)	7.21	7.52	9.48

Table 3. Calibration graph results for intra- and inter-assay validation runs

Run No.	Standard concentration (pg ml^{-1}) 200	300	400	600	800	1000
1	205	292	415	569	792	1027
2	195	300	422	590	775	1018
3	197	305	398	607	793	999
Mean	199	299	411	589	786	1015
RSD (%)	2.57	2.24	2.96	3.21	1.29	1.4
DEV (%)	−0.57	−0.29	2.88	−1.83	−1.68	1.49

represent the QC intra- and inter-assay summary for the QC validation experiments. The intra-assay precision (within-run RSD) and inter-assay precision (between-run RSD) were found to be $<10\%$ for each QC concentration (Table 2). The overall RSD was also $<10\%$ (Total precision, Table 2). In addition, the inter-assay precision calculated from the calibration standards did not exceed 4% for any calibration level (Table 3). These measures indicated acceptable intra- and inter-assay precision for the assay procedure.

Table 2 also presents the accuracy results for the QC samples. The DEV of the grand mean calculated concentration from the theoretical (nominal) concentration was within -8% for each of the three QC levels. This indicated acceptable accuracy for the assay procedure. The tabulated summary results for the intra-assay precision and accuracy for the extended calibration range (0.2–10 ng ml^{-1}) are shown in Table 4. These data suggest acceptable accuracy for the assay procedure.

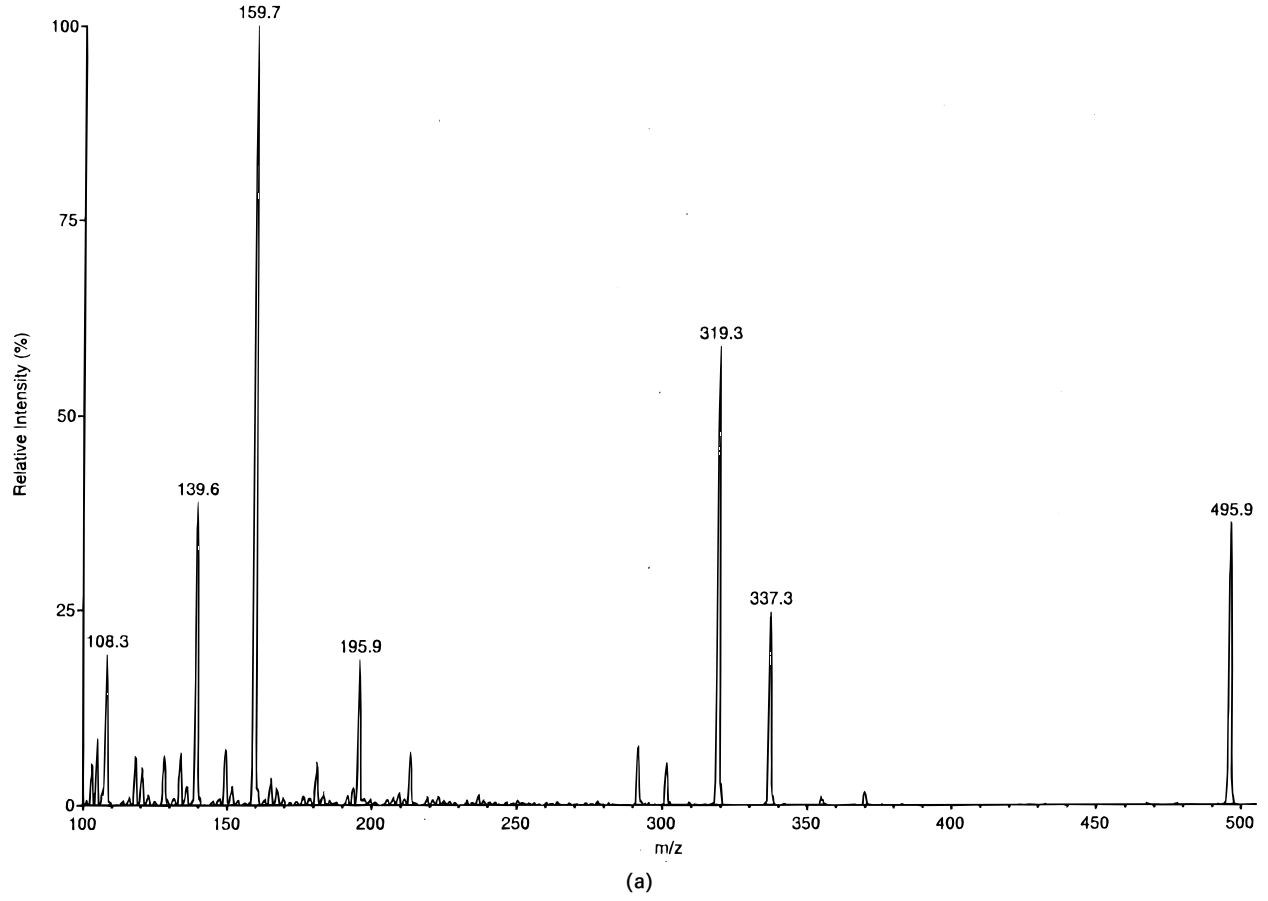
Stability

Information on the ability to store extracted samples over a period of time is beneficial to allow the greatest flexibility in sample processing and analysis. Several experiments to determine analyte stability were performed. These studies demonstrated Ro 18-0647 stability for up to 5 days in unreconstituted dry extracts stored at $-20\,^{\circ}C$ and for up to 24 h in reconstituted samples at ambient temperature. The DEV of the calculated concentrations of dry extracts ranged from -0.68 to -4.15% for 24 h samples and from 2.56 to 8.30% for 5 day samples. The RSD for these analyses

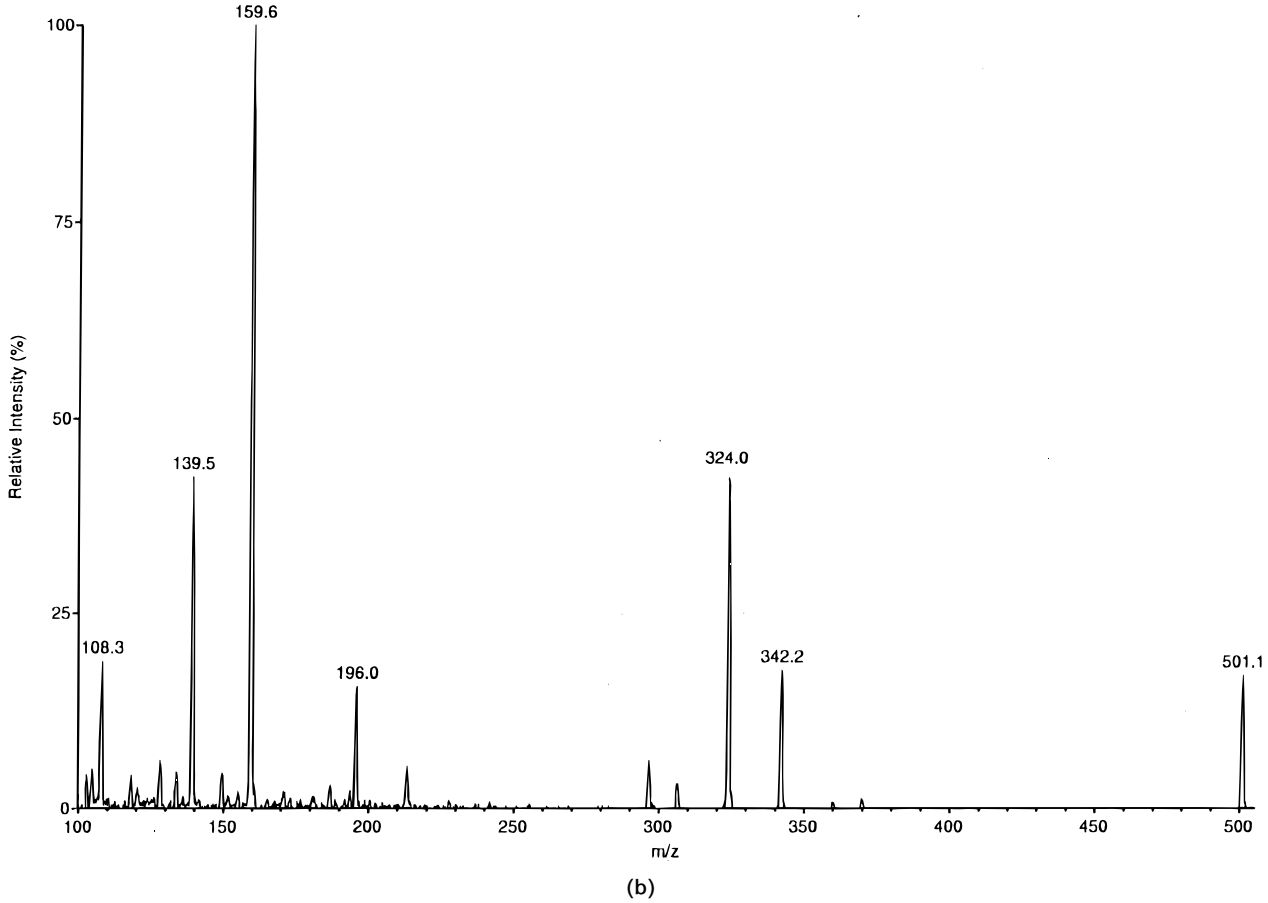
Table 4. Intra-assay precision and accuracy summary for extended curve range

	Nominal concentration (ng ml^{-1})		
Replicate	0.345	4.32	8.61
1	0.301	3.98	8.54
2	0.204[a]	4.29	8.90
3	0.300	4.26	9.10
4	0.333	4.23	8.88
5	0.312	4.30	8.25
Mean	0.318	4.21	8.73
SD	0.02	0.13	0.34
RSD(%)	6.29	3.13	3.86
DEV (%)	−7.77	−2.54	1.2

[a] Not used in calculations.



(a)



(b)

Figure 2. Full-scan collision-induced mass spectra of (A) the parent drug, Ro 18-0647 and (B) the internal standard, Ro 18-0647-d_5.

were within 8%. The desire to have unattended, robotic analysis of large batches of samples requires stability studies of Ro 18-0647 in the reconstitution solution. The DEV of the mean calculated concentration of Ro 18-0647 from the expected value for the 24 h samples was within −7.92% compared with −0.96% for the 0 h samples. This represents a decrease of ~7% over a 24 h period. This is within the generally accepted 15% which would indicate instability. The RSD for the 0 and 24 h analyses were <11%.

The stability of Ro 18-0647 in human plasma was also investigated. Ro 18-0647 is stable in plasma for at least 3 h at ambient temperature (~25 °C). The DEV from the expected concentration for 3 h samples was −7.09% compared with −0.96% for freshly prepared and extracted ($t = 0$ h) samples. However, the mean concentration of Ro 18-0647 after 6 h ($t = 6$ h) had a DEV of −16.39% from the expected concentration, indicating that Ro 18-0647 is not stable in plasma under these conditions for 6 h. The concentration of Ro 18-0647 in plasma was determined to be unchanged after 16 days of storage at −70 °C. The RSD for the experiment was <7%. Ro 18-0647 demonstrated stability in plasma to four cycles of freezing (−70 °C) and thawing to room temperature. The experiment was intended to reproduce the conditions under which plasma samples may be subjected if reanalyses are required. The DEV from theoretical was <5%. The largest negative DEV was −1.25%, indicating the stability of Ro 18-0647 to freezing and thawing. The RSD for this experiment was <9%.

DISCUSSION

The full-scan collision-induced dissociation (CID) mass spectra for Ro 18-0647 and Ro 18-0647-d_5 displayed abundant ion current signals for the protonated molecule ions $[M + H]^+$ (Fig. 2). Ammonium ion adducts $[M + NH_4]^+$ were also present in the full-scan single mass spectra with approximately equivalent relative abundances (unpublished results). The full-scan tandem mass spectra of Ro 18-0647 and Ro 18-0647-d_5 gave product ions from the $[M + H]^+$ precursor ions that are shown in Fig. 2. These spectra are dominated by an abundant product ion at the nominal mass of m/z 160 for both the parent drug and the deuterated internal standard. In contrast, both m/z 319 and 337 for the parent drug shift 5 Da in mass to give m/z 324 and 342 for the pentadeuterated internal standard [Fig. 2(B)] indicating that the five deuterium atoms are retained in these fragments.

Initially, we evaluated LC/MS/MS experiments under SRM conditions of the respective ammonium adducts to product ions at m/z 496 for Ro 18-0647 and at m/z 501 for Ro 18-0647-d_5. However, as assay development progressed to the point of quantifying Ro 18-0647 concentrations of 0.05–0.2 ng ml^{-1} in extracted plasma, a co-eluting component was observed in plasma blanks. Furthermore, a peak eluting later than Ro 18-0647 was also observed. This later eluting peak, combined with the requirement for high sample throughput, precluded the separation of the peak of interest and the co-eluting interference. Therefore, SRM of the respective protonated molecule ions to their product ions at m/z 160 for Ro 18-0647 and Ro 18-0647-d_5 were monitored (Fig. 2). Monitoring this reaction showed no evidence for chemical interference in the region of interest, and improved detection levels due to the high relative abundance of m/z 160 in each compound.

When SRM studies of the respective protonated ions fragmenting to m/z 160 for Ro 18-0647 and Ro 18-0647-d_5 were used, a lower level of detection of 0.1 ng ml^{-1} and a lower level of quantitation of 0.2 ng ml^{-1} was achieved. Although the method has demonstrated 0.025 ng ml^{-1} detection limits (Fig. 3), a routine lower level of quantitation (LLQ) of 0.2 ng ml^{-1} was required for the purposes of the described work. The 0.025 ng ml^{-1} detection limit was obtained by monitoring the SRM transition from the protonated molecule to an alternative product ion at m/z 319 for R0 18.0647 and R0 18-0647-d_5 internal standard using a Spherisorb C_6 HPLC column (2 mm i.d. × 50 mm). This alternative choice of m/z 319 as the preferred product ion for an improved LLQ was made to minimize the chemical noise at the retention time of 1.45 min where the target analyte eluted under these HPLC conditions (see Fig. 3). The initial portions of this work were performed on a PE SCIEX API III instrument, but later work was performed on the PE SCIEX API III$^+$ system. The improved collision cell of this latter system provides higher sensitivity in the MS/MS mode. Whereas an injection volume of 10 out of 30 µl of sample were injected initially, when the API III$^+$ is used an injection volume of 1–3 µl out of 50 µl is used to reach the 0.2 ng ml^{-1} LLQ.

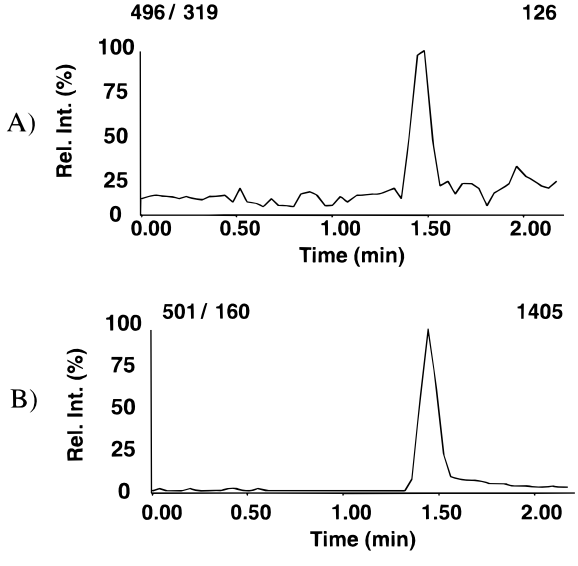


Figure 3. SRM LC/MS selected ion current chromatogram of a human plasma extract where the plasma had been spiked at the 0.025 ng ml^{-1} level. (A) Extract of human plasma that had been spiked with 0.025 ng ml^{-1} Ro 18-0647 prior to extraction. The major collision-induced fragmentation process from m/z 496 to 160 was monitored. (B) The same extract of human plasma that had also been spiked with 0.50 ng ml^{-1} of Ro 18-0647-d_5 prior to extraction. The major collision-induced fragmentation process from m/z 501 to 160 was monitored.

To contrast the analytical potential for combined LC/MS/MS techniques, control plasma extracts were analyzed by HPLC/UV detection. A control blank containing 0.5 ng ml^{-1} of the pentadeuterated internal standard and a plasma sample spiked with the same level of internal standard plus Ro 18-0647 at the 1 μg ml^{-1} level were extracted using the same extraction procedure as described in the Experimental section. The HPLC/UV analyses (220 nm) of these two extracts [Fig. 4(A) and (B)] are compared with the LC/MS/MS SRM

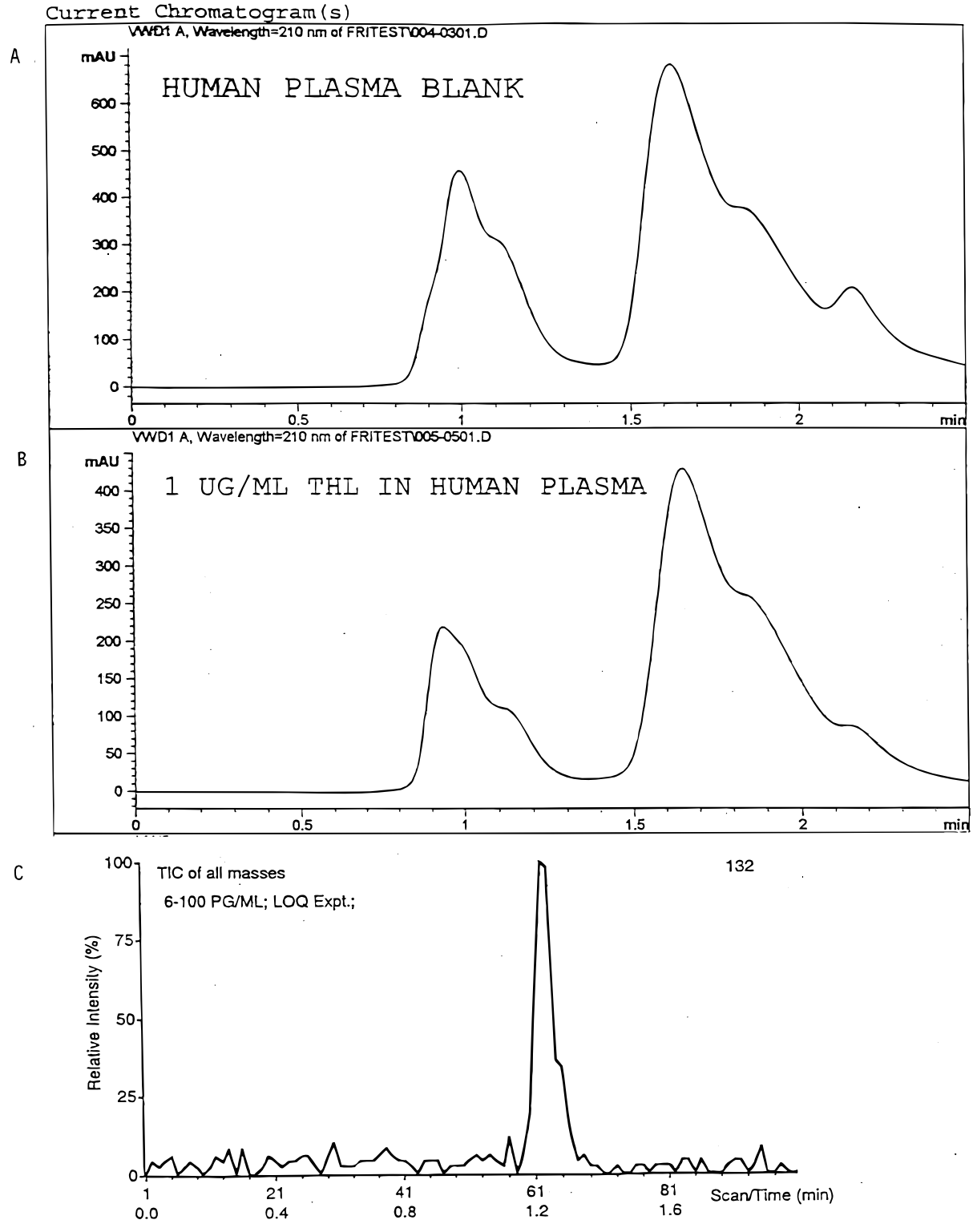


Figure 4. HPLC/UV and SRM LC/MS determination of Ro 18-0647 in human plasma. (A) HPLC/UV chromatogram at 220 nm of an extract of human plasma blank. (B) HPLC/UV chromatogram at 220 nm of an extract of human plasma that had been spiked with 1 μg ml^{-1} of Ro 18-0647 prior to extraction. (C) SRM LC/MS selected ion current chromatogram of an extract of human plasma that had been spiked with 0.10 ng ml^{-1} of Ro 18-0647 prior to extraction. See text for details.

analysis of another plasma extract obtained from a plasma sample spiked with Ro 18-0647 and the pentadeuterated internal standard spiked at 0.10 and 0.50 ng ml^{-1}, respectively [Fig. 4(C)]. As expected, no evidence for the parent drug is observed in the blank plasma extract in Fig. 4(A). However, Fig. 4(B), where the plasma sample contained 10^4 times more of the parent drug than was present in the sample shown in Fig. 4(C), shows no evidence for the parent drug. In fact, the HPLC/UV traces for the blank and the spiked sample cannot be distinguished [Fig. 4(A) and (B)] even though the analyte is present at a level far in excess than is required for the goals of this work. When 1 μg of an analytical standard of the parent drug is analyzed by LC/UV under the same conditions as used for the data shown in Fig. 4(A) and (B), the drug is easily detected (unpublished results). Thus the matrix components present in these samples appear to mask the analytes under these HPLC/UV conditions, but the very high specificity of LC/MS/MS experiments precludes this interference [Fig. 4(C)]. We[8–10] and others[11–14] have found that this capability is generally available for a wide variety of pharmaceutical and environmental applications. The specificity of LC/API MS/MS is so high that HPLC method development intended for bioanalytical work is best done on-line with MS to facilitate rapid method development and application.

Liquid–liquid extraction procedures using isooctane, *n*-butyl chloride and methylene chloride were investigated. None of the procedures resulted in less complex chromatograms or better recovery than hexane. For the purposes of this study, solid-phase extraction procedures using C_2, C_8, C_{18}, phenyl and CN stationary phases did not provide satisfactory recoveries. The following solutions were evaluated to determine the optimum conditions for precipitation of plasma proteins: acetonitrile, acetonitrile–water, acetone–water and trichloroacetic acid. A variety of acetonitrile–water and acetone–water combinations were used for sample treatment prior to solid-phase extraction. However, 100% acetonitrile protein precipitation followed by 100% hexane liquid–liquid extraction proved to be superior.

The preferred combination of extraction and HPLC/MS/MS described in this paper proved satisfactory for the stated goals. Figure 5 shows the SRM chromatogram for the LC/MS/MS analysis of a blank plasma extract [Fig. 5(A)] while simultaneously monitoring the *m/z* 496–160 transition for the protonated molecule ion of Ro 18-0647 and the corresponding *m/z* 501–160 transition for the pentadeuterated internal standard in the same plasma extract [Fig. 5(B)]. The internal standard was spiked into the plasma at the 0.50 ng ml^{-1} level. These data demonstrate that there are no detectable interferences in the plasma extract for the parent drug. An identical experiment demonstrated that there was also no interference for the internal standard in a plasma sample containing neither Ro 18-0647 nor Ro 18-0647-d_5 (unpublished results).

An example of a representative SRM LC/MS trace from the LLQ experiment is shown in Fig. 6. Figure 6(A) shows the selected ion current profile for the parent drug spiked into human plasma at the 0.20 ng ml^{-1} level. The *m/z* 496–160 transition for the parent drug clearly shows the expected chromatographic profile which co-elutes with its pentadeuterated internal standard shown in Fig. 6(B). A retention time of 0.95 min is observed for both the target analyte and its deuterated

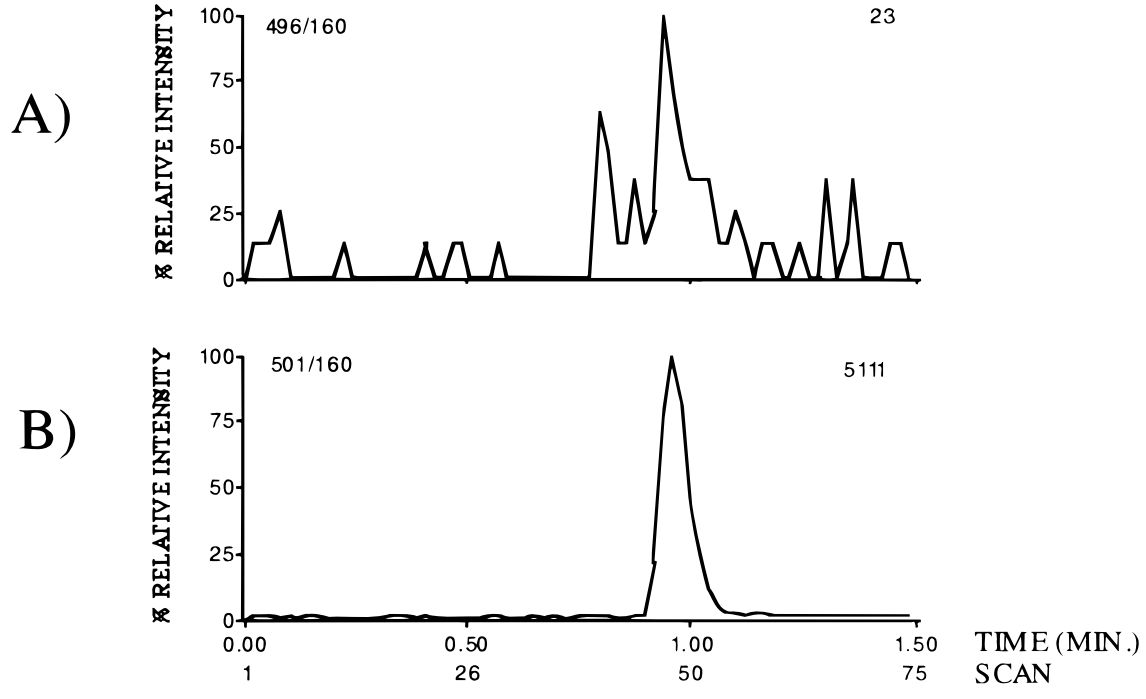


Figure 5. SRM LC/MS selected ion current chromatograms of a blank extract of human plasma. (A) Extract of human plasma that had not been spiked with Ro 18-0647 prior to extraction. The major collision-induced fragmentation process from *m/z* 496 to 160 was monitored. (B) The same extract of human plasma that had been spiked with 0.50 ng ml^{-1} of Ro 18-0647-d_5 prior to extraction. The major collision-induced fragmentation process from *m/z* 501 to 160 was monitored.

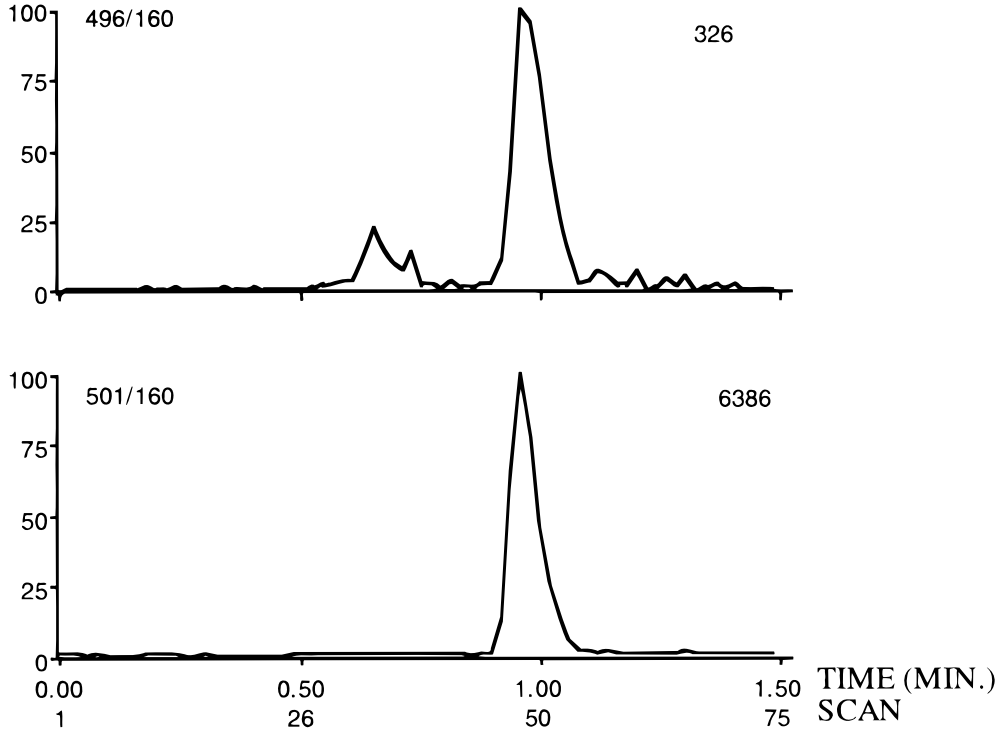


Figure 6. SRM LC/MS selected ion current chromatograms of an LLQ extract of human plasma. (A) Extract of human plasma that had been spiked with 0.20 ng ml^{-1} Ro 18-0647 prior to extraction. The major collision-induced fragmentation process from *m/z* 496 to 160 was monitored. (B) The same extract of human plasma that had also been spiked with 0.50 ng ml^{-1} of Ro 18-0647-d_5 prior to extraction. The major collision-induced fragmentation process from *m/z* 501 to 160 was monitored.

internal standard, as expected for experiments carried out under identical chromatographic experimental conditions. The slight differences in retention time behavior observed in these data compared with those shown in Figs 3 and 4 are due to minor variations in eluent and chromatographic conditions used in these experiments.

As of this writing, over 5000 clinical samples have been analyzed using the described method by several scientists in this laboratory. This analytical approach allows the routine quantitative determination of sub-nanogram levels of the parent drug from a wide variety of patients and treatments. More recently, the method has been applied to the analysis of rat, dog and monkey plasma samples with minimal changes in the procedure. In the case of the rat, sample sizes as small as 0.1 ml may be quantified. This procedure has met the goals of the study and proved to be useful and analytically rugged for routine applications in the analysis of large numbers of plasma samples.

CONCLUSIONS

This LC/MS/MS assay procedure for Ro 18-0647 in human plasma has proven to be sensitive, specific, accurate and reproducible. Its high sensitivity allows reliable and reproducible quantitation of Ro 18-0647 at the 0.20 ng ml^{-1} level in plasma. The high specificity of the assay allows fast chromatography and a relatively high throughput of ~50 injections per hour. Assuming an 8–10 h day of automated LC/MS/MS analyses, it is possible to analyze 400–500 samples per day on one instrument. The challenge for LC/API MS/MS analyses such as described in this paper is sample preparation procedures that are capable of preparing samples fast enough for the HPLC/MS system. Thus the bottleneck in this and related work is not the LC/MS/MS step, but rather the sample preparation stage. Although the sample preparation in this case is simple with only a protein precipitation step followed by a single liquid–liquid extraction, it would be preferrable to automate this aspect of the work to handle large numbers of samples efficiently.

A quantitative LC/MS/MS procedure has been developed to meet the high sensitivity, specificity and sample throughput required for the analysis of a large numbers of clinical plasma samples for Ro 18-0647. Significant gains have been made compared with previous procedures and it has been shown that LC/API MS/MS techniques are well suited for modern bioanalytical applications important to the pharmaceutical industry.

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