ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 1215-1223

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of a glycine/NMDA receptor antagonist in human plasma and urine using column-switching high-performance liquid chromatography with ultraviolet, fluorescence and tandem mass spectrometric detection ☆

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Received for review 23 September 1994; revised manuscript received 23 January 1995

Abstract

High-performance liquid chromatography (HPLC) assays using ultraviolet (UV) and fluorescence (FL) detection were developed and compared with a liquid chromatography/tandem mass spectrometry (LC/MS-MS) method for determination of the glycine receptor antagonist 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1*H*)-quinolone (L-701, 324, **I**) in human plasma and urine. The drug and internal standard (**II**) were isolated from the biological matrix through liquid–liquid extraction. In the HPLC-UV and HPLC-FL methods, the samples were initially injected onto a Cyano BDS Hypersil column, and the chromatographic region containing the peaks of interest was heart-cut onto an analytical C-18 BDS Hypersil column via a column-switching device. The analyte was quantified by monitoring either absorbance at 226 nm or fluorescence at 385 nm following 230 nm excitation. The limit of quantitation for **I** extracted from 1 ml of plasma or urine was 5 ng ml^{-1}, and the assays were validated in the concentration range of 5–200 ng ml^{-1}. The LC/MS-MS method also utilized a column-switching protocol and was validated in the concentration range of 1–200 ng ml^{-1}. Both assays provided data with precision and accuracy within less than 10% for all points in the standard curve range.

1. Introduction

The majority of analytical methods for the determination of drug concentrations in biological fluids are based on high-performance liquid chromatography (HPLC) with ultraviolet (UV)/visible absorbance detection. For some compounds, it is possible to increase assay sensitivity and selectivity by detecting the analytes using fluorescence detection (for selected latest examples see Refs. [1]–[8]). Fluorescence (FL) is usually more sensitive than absorbance detection, and selectivity is enhanced owing to a decrease in the number of fluorescent endogenouse interferences present in the biological fluid extracts. Although many drugs are intrinsically fluorescent, a low fluorescence quantum efficiency may result in insignificant improvement of analyte quantitation using commercially available detectors.

Recently, tandem mass spectrometric (MS-MS) detection in combination with HPLC was shown to provide a significant breakthrough in the analysis of drugs in biological matrices [9–13]. Using atmospheric pressure chemical ionization and MS-MS detection, a number of

☆ Presented at the Ninth Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists (Analysis and Pharmaceutical Quality Section), San Diego, CA, 6–10 November 1994.

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SSDI 0731-7085(95)01535-3

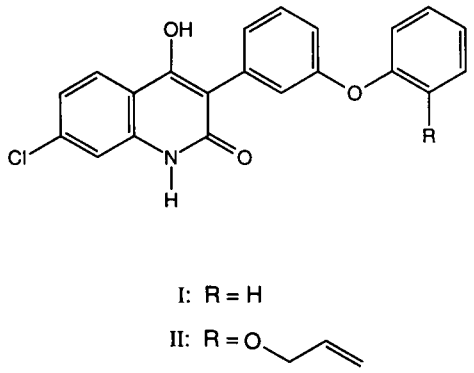

Fig. 1. Chemical structures of L-701,324 (I) and internal standard (II).

sensitive and selective analytical methods for various drug candidates in biological fluids have been developed [14–19].

7-Chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1*H*)-quinolone (Fig. 1, I) is an antagonist which acts at the glycine modulatory site on the *N*-methyl-D-aspartate (NMDA) receptor complex. This type of agent may be useful in the treatment of cerebrovascular diseases [20]. A sensitive and selective HPLC assay for the determination of I in plasma was required to support pharmacokinetic studies, and several HPLC approaches were evaluated to find the most sensitive and efficient method for the determination of I. The development of three methods based on HPLC with UV, FL, and MS-MS detection, and the comparison of assay performance using these methods is the subject of this paper.

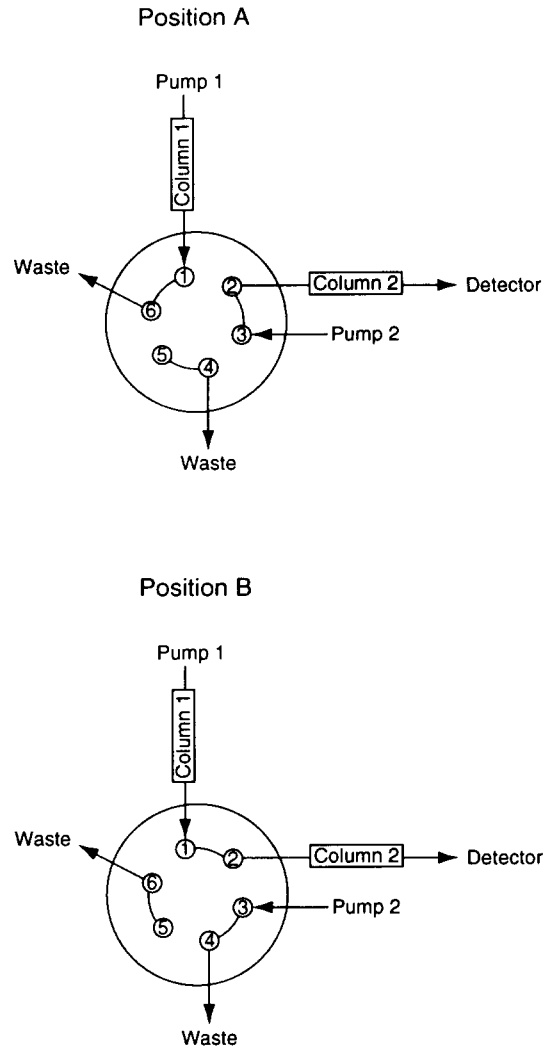

Fig. 2. HPLC system and the electronic switching valve positions used in the HPLC-UV and HPLC-FL assays.

2. Experimental

2.1. Materials

Compound I and an internal standard II (Fig. 1) were synthesized at the Merck Research Laboratories (Rahway, NJ, USA). All solvents (EM Science, Gibbstown, NJ, USA) and reagents (Fisher, Fair Lawn, NJ, USA) were of HPLC and analytical grade. Heparinized human control plasma was obtained from Sera-Tec Biologicals (North Brunswick, NJ, USA). Water was deionized using a Milli-Q reagent water system (Millipore, Milford, MA, USA) resulting in 18 MΩ conductivity. Solid-phase extraction (SPE) cartridges (C-2, C-8, C-18, cyano and phenyl, 1 ml) were purchased from Varian (Harbor City, CA, USA).

2.2. Instrumentation

HPLC-UV and HPLC-FL methods

Absorbance and fluorescence spectra were obtained using a model 8452A photodiode array spectrophotometer (Hewlett Packard, Palo Alto, CA, USA) and a model F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), respectively.

The HPLC system (Fig. 2) consisted of a series 410 LC pump (pump 1) (Perkin-Elmer, Norwalk, CT, USA), a model 6000A isocratic pump (pump 2) (Waters, Milford, MA, USA), a model WISP 715 autosampler (Waters), a model 785A absorbance detector (Applied Biosystems, Ramsey, NJ, USA) and a model LC 240 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA). The eluent from the ana-

lytical column (column 2) was directed into the absorbance detector, then into the fluorescence detector and finally to waste. A six-port-column-switching valve (Valco, Houston, TX, USA) was employed to divert the eluent from column 1 to waste or on to column 2. The switching valve was controlled via time event programming on pump 1. The times at which the events on pump 1 were set to trigger the valve were determined prior to analysis by injecting a standard solution of **I** and **II**, and monitoring the retention times of each compound eluting from column 1. The timed events were then set to place the valve in position A at the beginning of the run, switch the valve to position B at 4.6 min, and return the valve to position A at 6.6 min. The chromatographic data were acquired and analyzed with an automated laboratory system (PE/Nelson Access*Chrom V 1.7, Cupertino, CA, USA). Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted linear regression of the standard line. The standard line was constructed by plotting peak height ratios of **I** to **II** vs. drug concentration. All calculations were performed using PE Nelson Access*Chrom software.

The mobile phases for pumps 1 and 2 were composed of a 47:53 (v/v) and a 60:40 (v/v) mixture of acetonitrile (ACN) and phosphate buffer (10 mM; pH 3), respectively. The mobile phases were filtered through a nylon filter (0.2 μm) and each was delivered at a flow-rate of 1.0 ml min^{-1}. Column 1 was a Cyano BDS Hypersil column (150 × 4.6 mm, 5 μm) and was protected with a Cyano BDS Hypersil guard column. Column 2 was a C-18 BDS Hypersil column (250 × 4.6 mm, 5 μm) preceded by a C-18 BDS Hypersil guard column. All columns were purchased from Keystone Scientific (Bellefonte, PA, USA).

LC/MS-MS

Liquid chromatography/tandem mass spectrometry (LC/MS-MS) was performed using a Sciex model API III triple quadrupole mass spectrometer (Thornhill, Ont., Canada). The liquid chromatograph included a model ISS-200 autoinjector (Perkin-Elmer, Norwalk, CT, USA) and a model 250 HPLC pump (Perkin-Elmer), which delivered a mobile phase consisting of a 60:40 (v/v) mixture of ACN–formic acid (0.1%) at a flow-rate of 0.7 ml min^{-1}. A C-18 BDS Hypersil column (50 × 3 mm, 3 μm) was used as an analytical column and was protected with a C-18 BDS Hypersil guard column. Under these conditions the drug and the internal standard eluted at 1.75 and 2.15 min, respectively. A column switching valve similar to that shown in Fig. 2 was also employed here, but the analytical column was placed as column 1 and column 2 was not utilized. During the first 0.9 min following injection, the eluent from analytical column 1 was diverted to waste, followed by activation of the valve to position B and introduction of the column eluent into the MS-MS system. This technique eliminated the introduction of very polar compounds extracted from plasma into the MS-MS system, and allowed continuous instrument operation without a loss in assay sensitivity.

The LC system was interfaced to a mass spectrometer via a Sciex heated nebulizer probe held at 500 °C. The nebulizing air pressure and auxiliary flow-rate were 80 p.s.i. and 2.0 l min^{-1}, respectively. Gas-phase chemical ionization occurred via a corona discharge needle operating at 4 μA and positive ions were passed into the quadrupole mass analyzer via a 0.0045 inch aperature. The protonated molecules $(M + H)^+$ of the drug (m/z 364) and the internal standard (m/z 420) were drawn through the first quadrupole filter (Q1), with argon ion collision induced fragmentation occurring in Q2 (+40 eV, 550×10^{12} atoms cm^{-2}), and the product ions at m/z 253 and 378 for **I** and **II**, respectively, monitored via Q3. The orifice potential was set at +70 V and the electron multiplier at −3.7 kV. The curtain gas was ultra-high purity nitrogen flowing at a rate of 0.9 l min^{-1}. The detector electronics counted every tenth pulse with a dwell time of 400 ms.

Unknown sample concentrations were calculated in a similar manner to HPLC-UV and HPLC-FL assays, but here the standard line was constructed by plotting peak area ratios of **I** to **II** vs. drug concentration rather than peak height ratios vs. drug concentration. All calculations were performed using MacQuan software (PE-Sciex).

2.3. Standard solutions

Separate standard stock solutions (1 mg ml^{-1}) were prepared in methanol for both the drug and the internal standard. Dilutions were made using methanol to give a series

of working standards with concentrations needed to construct a calibration curve.

2.4. Sample preparation

HPLC-UV and HPLC-FL methods

Calibration curves were constructed in control plasma and urine by adding 100 μl of the corresponding working standard of both **I** and **II** to 1 ml plasma (or urine) contained in 15 ml centrifuge tubes for analyte concentrations of 5, 10, 20, 40, 100, and 200 ng ml^{-1}, and an internal standard concentration of 50 ng ml^{-1}. The pH of the solution was controlled by adding 0.1 M acetate buffer (pH 5) to the centrifuge tube prior to extraction with 5 ml methyl *t*-butyl ether (MTBE). The drug and internal standard were back-extracted from the organic layer into 1 ml 0.1 N NaOH. After acidifying the aqueous layer with 500 μl 0.5 N HCl, both **I** and **II** were back-extracted into 5 ml MTBE, the organic layer was separated and the solvent was evaporated under a stream of nitrogen at 35 °C. The residue was reconstituted into 200 μl of mobile phase and 150 μl of this solution was injected into the chromatographic system.

LC/MS-MS

Plasma standards were prepared by adding 100 μl of the corresponding working standard of both **I** and **II** to 1 ml plasma for analyte concentrations of 1, 2, 5, 10, 50, 100, 150, 200 ng ml^{-1} and 50 ng ml^{-1} of internal standard.

Owing to the high selectivity of the LC/MS-MS assay, a multi-step liquid–liquid extraction was not necessary. After the drug and the internal standard were extracted from pH 5-buffered plasma with 5 ml MTBE, the organic solvent was evaporated to dryness, the residue was reconstituted in 300 μl of the mobile phase, and 100 μl was injected into the LC/MS-MS system.

2.5. Precision, accuracy, recovery, specificity, and stability

The precision of the method was determined by the replicate analyses ($n = 5$) of human plasma and urine containing **I** at all concentrations utilized for constructing calibration curves. The accuracy of the method was expressed by (mean observed concentration)/(spiked concentration) × 100. The recovery was determined by comparing the peak area of **I** extracted from biological fluids to that of standards injected directly. Assay specificity was assessed by assaying blank control plasma and urine from five different sources. No endogeneous interferences were observed.

The stability of the drug in plasma was determined by spiking control plasma with **I**, resulting in final concentrations of 10 and 150 ng ml^{-1}. These solutions were aliquoted into polypropylene tubes, capped, and kept at −20 °C. Five replicate samples at each concentration were extracted and assayed on three separate days. Concentrations were determined from a calibration curve prepared from different lots of human control plasma on each day of analysis.

3. Results and discussion

3.1. HPLC-UV and HPLC-FL methods

The development of sensitive HPLC methods based on UV and FL detection required the optimization and evaluation of three distinct steps, including isolation of **I** and **II** from biological fluids, chromatographic separation of **I** and **II** from endogeneous impurities present in the biological fluid extracts, and sensitive detection of **I**.

Fluorescence and absorbance spectra of **I** in methanol were initially obtained to establish the most favorable conditions for the detection of **I**. The absorbance spectrum of **I** indicated the presence of three absorption bands with maxima at 226, 284, and 322 nm, and molar absorption coefficients of 67 500, 13 500, and 13 700 M^{-1} cm^{-1}, respectively. Based on these data, 226 nm was chosen as the detection wavelength in the HPLC-UV assay. The fluorescence emission spectrum indicated the presence of a band with a maximum at 380 nm. These wavelengths were used initially as a guide in the optimization of the HPLC fluorescence detector utilized in the assay. Using this detector, emission with the greatest signal-to-noise ratio was observed when 230 and 385 nm were used as the wavelengths of excitation and emission, respectively, and these wavelengths were employed in the HPLC-FL assay. Fluorescence detection was used concurrently with absorbance detection to determine if greater selectivity and/or sensitivity could be obtained via fluorescence vs. UV detection of **I**. The results

obtained indicated that the limit of quantitation and assay sensitivity was practically the same using both UV and fluorescence detection.

Isolation of **I** from plasma was attempted using both liquid–liquid and SPE. Initially, SPE on a variety of reversed-phase cartridges, including C-2, C-8, C-18, cyano and phenyl, was evaluated. After precipitation of plasma protein with ACN (1 ml), the supernatant was diluted with pH 3 phosphate buffer (0.1 M; 5 ml) and loaded onto cartridges pre-conditioned with methanol and 0.1 M phosphate buffer (pH 3). The cartridges were washed with water followed by 50% aqueous methanol, 35% aqueous iso-propanol and finally hexane. The sample was eluted from the cartridges with methanol (1 ml), and after evaporation of the organic extract to dryness and reconstitution of the residue in the mobile phase (200 µl), the solution (150 µl) was injected into the HPLC system. The mean recovery of **I** from plasma was approximately 90%. However, several impurities which interfered with the integration of the peaks of interest remained in the extracts. In addition, the SPE required a number of time-consuming steps, making the sample preparation procedure highly inefficient.

Therefore, isolation of **I** from plasma using liquid–liquid extraction was attempted. Several extraction solvents were evaluated including hexane, MTBE, toluene, ethyl acetate, and various mixtures of toluene–ethyl acetate (80:20 and 60:40, v/v), and toluene–ethyl acetate–2-propanol (50:49:1, v/v). The MTBE extracts contained a number of components from plasma which coeluted with **I** and **II**, and were detected by both the absorbance and fluorescence detectors. The pK_a of **I** is 9.5, and the extraction efficiency should be greatest at pH $\leqslant$ 7, which was experimentally confirmed. Attempts were made to eliminate endogenous interferences by varying the pH of plasma to 1, 3, 5, and 6 prior to extraction. The highest recovery was obtained when the plasma pH was 5. After initial extraction at pH 5, the endogenous impurities were removed by back-extraction into base, followed by acidification and subsequent extraction into MTBE. After evaporating the MTBE extracts to dryness, the residue was reconstituted in the mobile phase (200 µl) and part of this solution (150 µl) was injected into the HPLC system.

Chromatography was initially performed without column-switching using a single C-18 BDS Hypersil (150 × 3 mm, 3 µm) column and ACN–phosphate buffer (10 mM; pH 3) (47:53, v/v) as the mobile phase. The chromatographic peak for **I** was rather wide (peak-width-at-half-height was approximately 18 s) and the limit of quantitation (LOQ), defined as the lowest point on the standard curve for which assay accuracy and precision was within less than 10%, was only 12.5 ng ml^{-1} (data not shown). The peak compression necessary to improve assay sensitivity was accomplished via application of a column-switching technique. The extracted sample was delivered to a Cyano BDS Hypersil

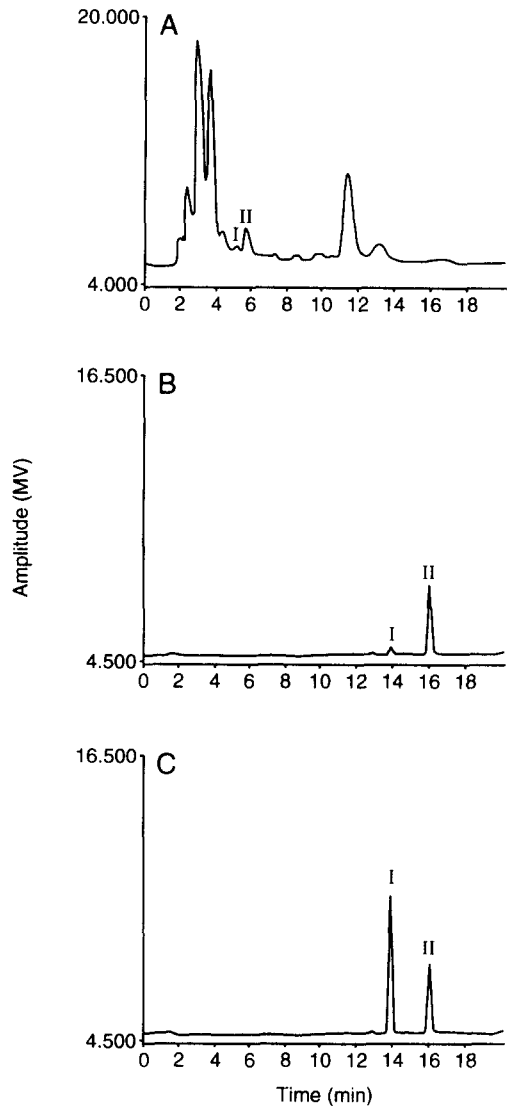

Fig. 3. Chromatograms of **I** and **II** extracted from human-control plasma and detected at 226 nm; (A) 10 ng ml^{-1} of **I** and 50 ng ml^{-1} of **II** eluting from column 1, (B) 5 ng ml^{-1} of **I** and 50 ng ml^{-1} of **II** eluting from column 2 after column-switching, (C) 100 ng ml^{-1} of **I** and 50 ng ml^{-1} of **II** eluting from column 2 after column-switching. For experimental conditions see text.

column (column 1, Fig. 2) and the eluent containing the chromatographic peaks of interest was heart-cut to a C-18 BDS Hypersil column, as outlined in the Experimental section. A typical chromatogram obtained using absorbance detection of the eluent from column 1 before transferring to column 2 is shown in Fig. 3(A). The drug and internal standard were only partially separated from each other and from plasma endogeneous interferences. Further separation was obtained when the eluent from column 1 was directed onto column 2, as shown in Figs. 3(B) and 3(C). Using this technique, the assay was validated in the concentration range of 5–200 ng ml^{-1}. The LOQ was practically the same for both fluorescence and absorbance detection, owing to the relatively low flourescence intensity and high absorbance ($\epsilon = 67\,500$ M^{-1} cm^{-1} at 226 nm) of **I**. The precision and accuracy data for the HPLC-UV and HPLC-FL assay in plasma and urine are presented in Table 1.

The intraday precision, defined as the relative standard deviation (RSD), at all concentrations on the standard line was less than 10%, and the accuracy was within 95–109%. Interday stability data were obtained by assaying quality control samples containing 10 and 150 ng of **I** added to 1 ml of human-control plasma. Five replicate samples at each concentration were extracted and assayed on three consecutive days. All measured concentrations were within 20% of the nominal values with a precision better than 10%.

Assay specificity was achieved through a multi-step sample preparation procedure which effectively removed the endogenous interferences from plasma. Specificity of the assay was confirmed by assaying plasma extracts from five different lots of plasma. No endogeneous interferences were observed. The recovery of the drug from plasma was about 90% at all concentrations within the standard curve range.

3.2. LC/MS-MS method

Sample preparation for the LC/MS-MS assay was simplified and based on a single liquid–liquid extraction of **I** and **II** from buffered plasma (pH 5) using MTBE. Quantitation involved monitoring the product ions of the drug and internal standard at m/z 253 and 378 originating from the protonated molecules at m/z 364 and 420, respectively (Fig. 4). Initially, method validation was attempted using direct injection of plasma extract into the LC/MS-MS system without column switching. However, after assaying about 100 plasma samples, a significant decrease in sensitivity was observed. This loss in sensitivity was probably due to adsorption of plasma endogenous components onto the surface of the orifice and/or the interface plate of the mass spectrometer. This resulted in a reduction in the instrumental response by about 30% over a series of 100 injections. To eliminate this loss in sensitivity, an on-line sample clean-up using a column-switching technique was employed, as described in the Experimental section.

Typical chromatograms obtained using this method are shown in Fig. 5. The assay precision and accuracy data presented in Table 2 indicate the intraday precision was $\leqslant 10\%$ at all concentrations on the standard line, and the assay accuracy was within 96–105%.

Table 1
Precision [a] and accuracy [b] data for the determination of **I** in human plasma and urine using HPLC-UV and HPLC-FL

Conc. (ng ml^{-1})	UV detection				Fluorescence detection			
	Plasma		Urine		Plasma		Urine	
	RSD (%) [a]	Accuracy [b]	RSD (%) [a]	Accuracy [b]	RSD (%) [a]	Accuracy [b]	RSD (%) [a]	Accuracy [b]
5	4.2	101	1.6	101	3.4	101	3.5	100
10	4.4	100	2.5	105	6.8	103	1.6	105
20	2.7	103	2.0	108	8.9	101	4.4	107
40	3.7	98	6.4	99	5.7	109	6.1	100
100	3.1	98	5.8	99	3.3	104	5.0	95
200	4.1	102	1.1	107	3.1	104	0.8	106

[a] Precision expressed as the relative standard deviation (RSD) $n = 5$.
[b] Accuracy expressed as (mean calculated conc.)/(spiked conc.) $\times 100$.

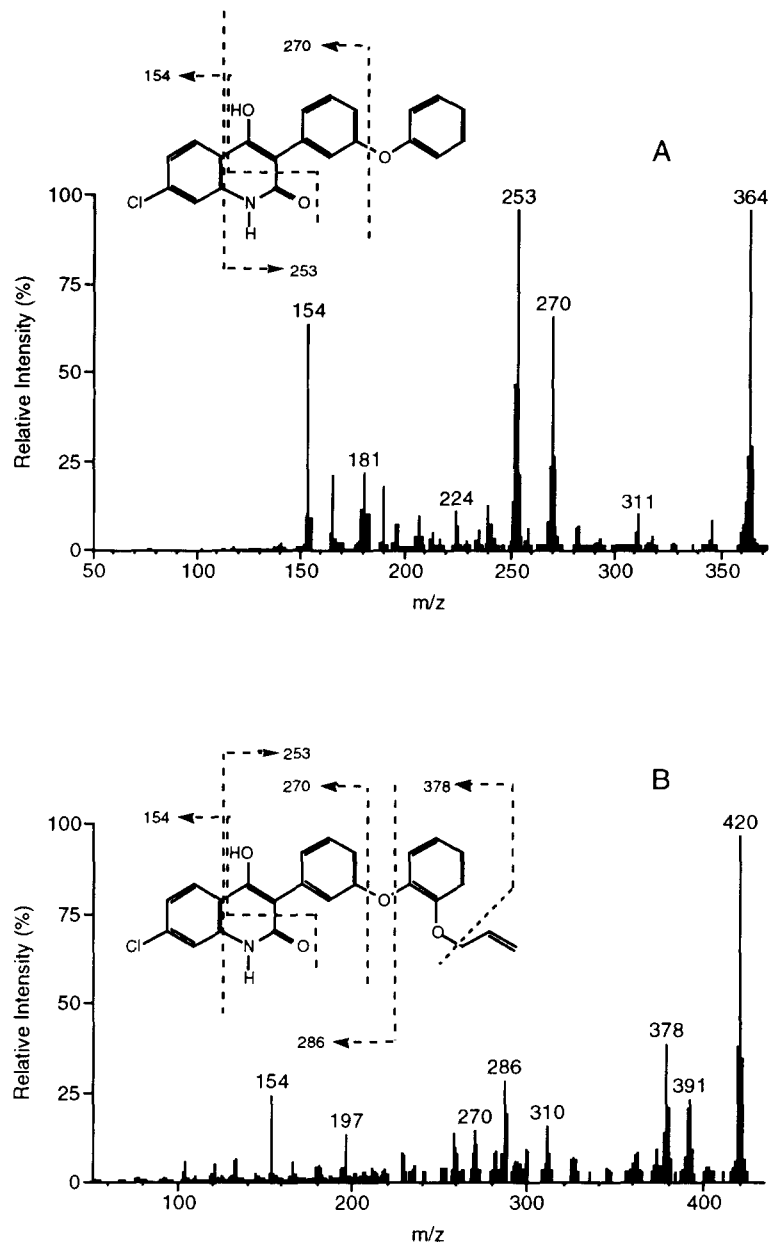

Fig. 4. Positive ion product mass spectra of the protonated molecules; (A) I (*m/z* 364), (B) II (*m/z* 420).

3.3. Comparison of HPLC-UV and HPLC-FL methods with HPLC/MS-MS

The LOQ for the assay of I in plasma using LC/MS-MS was 1 ng ml^{-1}, which was five times lower than that using the HPLC-UV or HPLC-FL methods. About 33% of the plasma extract was injected into the LC/MS-MS assay vs. the 75% needed in the HPLC-UV and/or HPLC-FL based methods. The high specificity of MS-MS detection also allowed simplification of sample preparation and chromatography. A single extraction step was sufficient for sample preparation in the LC/MS-MS assay in contrast to the multi-step liquid–liquid extraction, which was necessary to remove endogenous interferences in the HPLC-UV and HPLC-FL assays. Additional sample clean-up was needed in the UV or FL based assays, and was performed on-line using two chromatographic

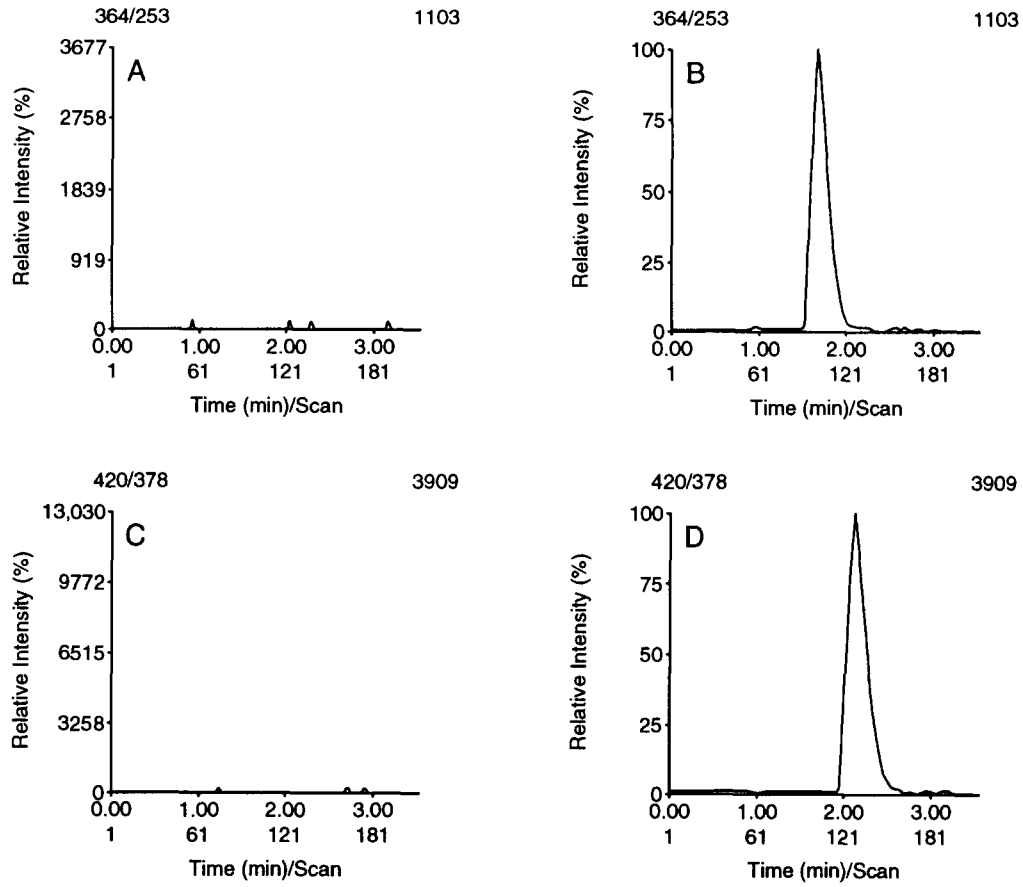

Fig. 5. Representative LC/MS-MS chromatograms of plasma extracts obtained by multiple reaction monitoring at m/z 364 → 253 for **I**, and m/z 428 → 378 for **II**; chromatograms A and C are extracts of blank plasma; chromatograms B and D are extracts of plasma spiked with 10 ng ml^{-1} of **I** and 50 ng ml^{-1} of **II**, respectively. The numbers in the upper right-hand corner of the chromatograms indicate the peak height expressed in arbitrary units.

columns and by "heart-cutting" the peaks of interest from the first column to the second column using column-switching methodology. A column-switching technique was also used in the LC/MS-MS assay, but the procedure was greatly simplified and involved only one column. The analysis time of the LC/MS-MS assay was only 3 min, and was considerably shorter than the analysis times (22 min) of the HPLC-UV or HPLC-FL assay. The simplified extraction procedure combined with the short chromatographic run time allowed analyses of more then 150 samples per day using the LC/MS-MS assay vs. 40–50 samples using HPLC-

Table 2
Validation data for the determination of **I** in human plasma using LC/MS-MS

Replicate analysis	Concentration (ng/ml^{-1})							
	1	2	5	10	50	100	150	200
1	0.9	2.1	5.1	9.6	44.9	96.2	139.9	195.0
2	1.1	2.0	5.1	10.3	48.8	103.5	156.5	195.3
3	0.9	2.2	5.1	10.3	49.2	108.7	152.5	202.1
4	1.0	1.9	5.2	10.0	51.3	108.4	150.4	200.0
5	1.1	2.2	4.8	9.6	46.6	103.8	154.4	198.3
Mean	1.0	2.1	5.1	10.0	48.2	104.1	150.7	198.1
RSD (%) [a]	10.0	6.3	3.0	3.5	5.1	5.1	4.3	1.5
Accuracy [b]	100	105	102	100	96	104	101	99

[a,b] As defined in Table 1.

UV or HPLC-FL methods. Also, the flow-rate was lower (0.7 ml min^{-1}) in the LC/MS-MS assay compared with the UV or FL based methods (1 ml min^{-1}). The lower flow-rate and an almost seven-fold decrease in run time allowed substantial savings in the use and disposal of organic solvents.

4. Conclusion

The utility of LC/MS-MS as a sensitive and selective analytical technique for the determination of trace quantities of drugs in biological fluids has been confirmed. Selectivity enhancement obtained using tandem mass spectrometric detection simplified sample extraction procedure and chromatography, and led to an improvement in assay sensitivity in comparison with conventional methods based on HPLC with UV and FL detection.

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