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Journal of Chromatography A, 712 (1995) 75–83

JOURNAL OF CHROMATOGRAPHY A

Determination of an endothelin receptor antagonist in human plasma by narrow-bore liquid chromatography and ionspray tandem mass spectrometry

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Abstract

A method is described for the determination of a new endothelin receptor antagonist, Bosentan Ro 47-0203, in human plasma using narrow-bore liquid chromatography and ionspray tandem mass spectrometry. After protein precipitation with acetonitrile, the compounds were extracted with dichloromethane at pH 11. The compounds were chromatographed on a 2 mm I.D. reversed-phase column and introduced into the mass spectrometer with an ionspray (pneumatically assisted electrospray) interface at a flow-rate of 170 μl/min without postcolumn splitting. Two different internal standards were used for the assay: either a structural analogue or a deuterated analogue. The limit of quantification was 0.5 ng/ml using a 0.5-ml aliquot of plasma. Concentrations of the drug were determined in the range 0.5–200 ng/ml. The recovery from human plasma was 87%. The new API IIIplus collision cell was about five times more sensitive than the original API III cell. The assay was demonstrated to be sensitive, selective and robust for the analysis of over 1500 samples.

1. Introduction

Liquid chromatography is widely used for the determination pharmaceutical compounds with UV, fluorescence or electrochemical detection. More recently, mass spectrometry (MS) has been introduced as a highly specific and sensitive detector for HPLC. Several approaches have been used to combine LC and MS, including direct liquid introduction (DLI) [1], thermospray (TS) [2], particle beam (PB) [3] and more recently electrospray [4] and ionspray [5]. For quantitative analysis, LC–MS, in particular with atmospheric pressure ionization (API), is rapidly becoming a powerful analytical tool with respect to sensitivity and selectivity compared with HPLC with UV detection. Ionspray can handle flow-rates from a few nanolitres per minute [6] to over 1000 μl/min [7]. For quantitative ionspray mass spectrometry, narrow-bore HPLC columns of 2 mm I.D. are a practical alternative to standard-bore columns owing to the lower solvent consumption and increased analyte response with good chromatographic performance [8].

Endothelin is a bicyclic 21-amino acid peptide and a very potent long-lasting vasoconstrictor. It is also a growth factor for vascular smooth muscle and mesangial and cancer cells and is a

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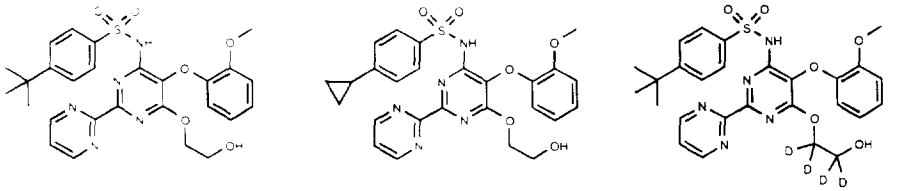
0021-9673/95/$09.50
SSDI 0021-9673(95)00331-2



Ro 47-0203 (I) Ro 47-8761 (II) Ro 47-0203/d4 (III)

Scheme 1. Structures of compounds **I–III**.

potent bronchoconstrictor [9–11]. A non-peptidic endothelin receptor antagonist, Bosentan [12] (Ro 47-0203, Scheme 1), is currently under development for the treatment of diseases such as hypertension, sub-arachnoid haemorrhage and Raynaud's syndrome. For pharmacokinetic studies, an HPLC–UV assay [13] was developed, but was insufficiently sensitive for complete characterization of the kinetics in man. Therefore, it was necessary to validate a sensitive and robust assay with ionspray tandem mass spectrometry for the determination of the drug at low levels in human plasma.

2. Experimental

2.1. Chemicals

Ro 47-0203 (**I**, $M_r = 552$), Ro 47-8761 (**II**, $M_r = 536$) and Ro 47-0203/002 (**III**, $M_r = 556$) (Scheme 1) were provided by F. Hoffmann-La Roche (Basle, Switzerland). Dichloromethane was obtained from Fluka (Buchs, Switzerland) and was of analytical-reagent grade. Acetonitrile, ethanol, ammonium acetate, Titrisol buffer solution (pH 11) and acetic acid were obtained from Merck (Darmstadt, Germany) and were of chromatographic or pro analysis grade. Water was doubly distilled in-house or was of chromatographic grade obtained from Merck.

2.2. Liquid chromatography

The mobile phase, acetonitrile–5 m*M* ammonium acetate–acetic acid (75:25:1 or 70:30:1, v/v), was delivered by a Model 6200A LC pump (Merck–Hitachi, Tokyo, Japan). The extracts were injected with an AS 4000 autosampler (Merck–Hitachi) equipped with a 100-μl injection loop on to a 125 × 2 mm I.D. column with a 10 × 2 mm I.D. guard column (Superspher RP-18, 4 μm; Stagroma, Wallisellen, Switzerland). The column temperature was held constant at 35°C using a Jones Chromatography (Llanbradach, UK) Model 9730 column thermostat. The mobile phase was degassed on-line using an SDU 2003 solvent degasser unit (Labsource, Basle, Switzerland).

2.3. Mass spectrometry

A Perkin-Elmer SCIEX (Thornhill, Canada) API III triple-quadrupole biomolecular mass analyser or the same instrument upgraded to a Perkin-Elmer SCIEX API IIIplus triple-quadrupole biomolecular mass analyser was used. The original collision cell was an open type with a crossed-flow jet, which suffered from a significant energy spread. The improved confined collision cell requires less gas flow and uses improved optics, but operates at pressures about ten times higher than the old cell. This high pressure (<0.66 Pa) causes ions, under the influence of r.f. fields, to relax into a narrow beam with well defined kinetic energy, which results in better sensitivity and better fragment ion resolution [14].

The mass spectrometer was equipped with an in-house constructed articulated ionspray device. This articulated ion spray device uses an 80 cm × 100 μm I.D. × 500 μm O.D. stainless-steel capillary as a sprayer capillary. The tip of the

capillary was biased at approximately 60° and electropolished. This capillary was placed inside a 35 mm × 800 μm I.D. × 1.59 mm O.D. stainless-steel capillary used as the nebulizer capillary. The tip of this capillary was shrunk to 650 μm. The nebulizer gas (nitrogen, +99.999% purity) was introduced using a T-piece. The other end of the sprayer capillary was connected to a ca. 100 mm × 100 μm I.D. × 170 μm O.D. fused-silica capillary through a zero-dead-volume union. The fused-silica capillary used for electrical isolation was connected to the column outlet through a Swagelok connector. The mass axis of the mass spectrometer was calibrated with a standard solution of quaternary alkylammonium salts in acetonitrile at a flow-rate of 10 μl/min covering a molecular mass range from 200 to 600.

Infusion experiments were performed with a Harvard syringe pump. Prior to quantitative analysis, the resolution of the quadrupoles was tuned with 1 ng/μl Ro 47-0203 solution [acetonitrile–5 m*M* ammonium acetate–acetic acid (30:70:1, v/v/v)] to be better than 1 u at the peak half-height for Q1 on both devices, better than 2 u at the peak half-height for Q3 using the API III and better than 1 u at the peak half-height for Q3 using API IIIplus. Argon was used as the collision gas in Q2 with a collision gas thickness (CGT) of $500 \cdot 10^{12}$ atoms/cm^2 (API III) or with a CGT of $250 \cdot 10^{13}$ atoms/cm^2 (API IIIplus). The collision energy used was set at 50 eV (API III) or 35 eV (API IIIplus).

The LC–UV trace was recorded with a ABI (Ramsey, NJ, USA) Model 783A UV detector, equipped with 2.4-μl volume, 6-mm path length micro detection cell in-line between the column outlet and the articulated ionspray device. The wavelength was set at 270 nm.

Method development and routine work were carried out using the standard software Tune 2.4, RAD 2.5 and Mac Quant 1.3 (Perkin-Elmer SCIEX).

2.4. Preparation of standard solutions

Stock standard solutions of compound **I–III** were prepared by dissolving about 4 mg in 100 ml of ethanol. Aliquots of the stock standard solution were diluted with ethanol to provide working standard solutions. The plasma standards were obtained by spiking blank plasma (20 ml) with 100 μl of working solution, providing concentrations between 0.5 and 200 ng/ml of the analyte. The standards were divided into aliquots of 0.5 ml and stored deep frozen at −20°C until required for analysis.

The stability of analyte **I** was investigated by preparing control plasma samples at concentrations of 2.5 and 80 ng/ml. Aliquots were frozen and stored at −20°C. Fresh calibration samples were prepared to provide 100% values and the data indicated that compound **I** was stable for 7 months in human plasma under the conditions investigated.

2.5. Sample preparation

An aliquot of human plasma (0.5 ml) was mixed with 10 μl of internal standard (I.S.) solution containing 50 ng of Ro 47-0203/002 or Ro 47-8761 in ethanol. Acetonitrile (0.75 ml) was added to the sample, which was homogenized by vortex mixing. To complete protein precipitation, the sample was kept in a refrigerator at 5°C for 10 min. The supernatant was extracted with dichloromethane (7 ml) at pH 11 by shaking (20 min) on a rotating shaker (Heidolph, Kelheim, Germany). After centrifugation (5 min) and removal of the upper aqueous phase, the organic phase was transferred into a new tube and evaporated to dryness in a vacuum centrifuge (Savant, Farmingdale, NY, USA). To remove lipids, the extract was dissolved in acetonitrile (1 ml) and centrifuged (5 min). The sample was transferred to a conical tube (1.1 ml) and evaporated to dryness in a vacuum centrifuge. Prior to LC–MS analysis, the extract was dissolved in 100 μl of acetonitrile–5 m*M* ammonium acetate–acetic acid (30:70:1, v/v/v). Volumes of 30 or 90 μl were injected.

2.6. Calibration and calculations

Along with the unknown samples, quality control samples and ten plasma standards with

appropriate drug concentrations were processed as described above. The concentrations in all samples were determined using peak areas with a calibration graph obtained by weighted linear least-squares regression (weighting factor = $1/y^2$) of the peak-area ratios of the calibration samples.

3. Results and discussion

3.1. Analytical system and chromatography

Pharmacokinetic studies required the quantification of **I** at levels below 1 ng/ml. This sensitivity could not be achieved by the standard HPLC–UV method. However, flow injection analysis indicated a very good sensitivity for **I** with ionspray MS. In LC–MS, the mobile phase composition plays an important role regarding sensitivity, and one is restricted to the use of volatile buffers such as ammonium acetate and acetic acid. The chromatography of **I** is relatively uncritical on reversed-phase material, and the choice of a narrow-bore column was dictated by a high organic content of the mobile phase and a sharp peak shape. For interfacing LC and MS, two approaches are possible: the heater nebulizer which allows gas-phase (APCI) and ionspray, which is a condensed-phase ionization process. Ionspray was selected because it provided the best response for the analytes with the HPLC mobile phase used. API produces mainly charged protonated molecular ions, which can be

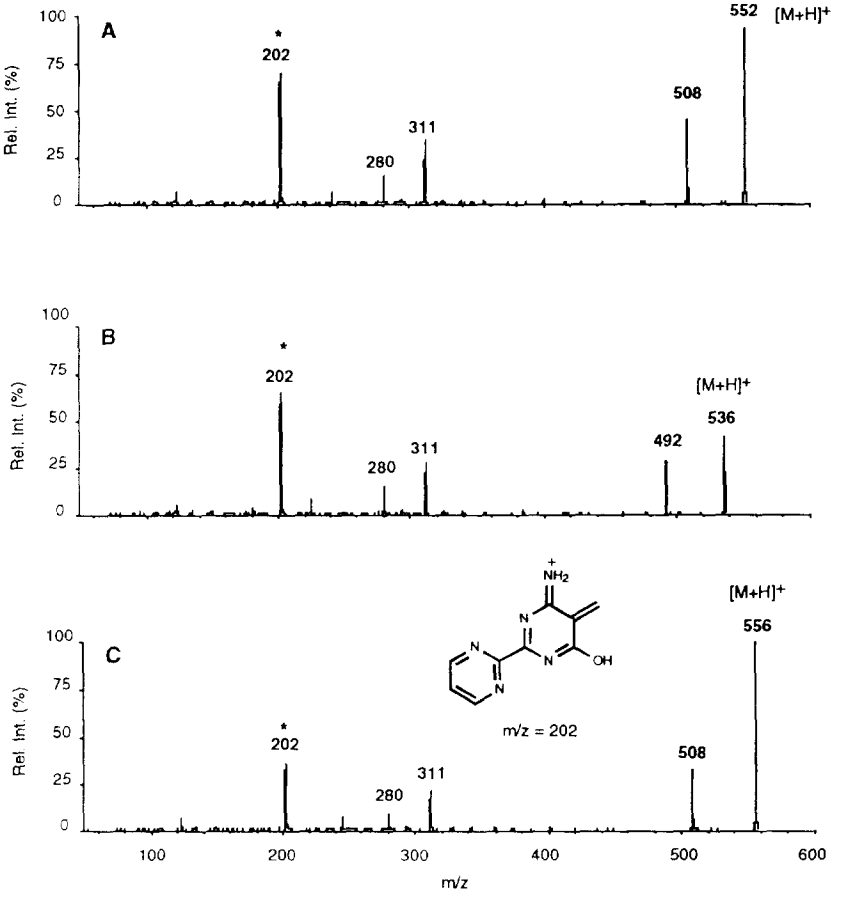


Fig. 1. Product ion spectra of (A) Ro 47-0203 (**I**), (B) Ro 47-8761 (**II**) and (C) Ro 47-0203/d4 (**III**) at a collision energy of 25 eV and a collision gas thickness of $300 \cdot 10^{13}$ atoms/cm^2.

fragmented in the collision cell (Q2) of a tandem mass spectrometer. Fig. 1 shows the product ion spectrum of **I** and the two internal standards **II** and **III**. For all three compounds, an intense product ion was observed at m/z 202 which was selected for the selected reaction monitoring (SRM) mode.

A major concern in standard HPLC–UV is co-elution of endogenous compounds with the analytes. Ro 47-0203 has a relatively intense chromophore, and a few nanograms of a standard solution can be detected easily. However, adequate selectivity could not be achieved with a plasma extract (2.5 ng/ml of **I** and 50 ng/ml of

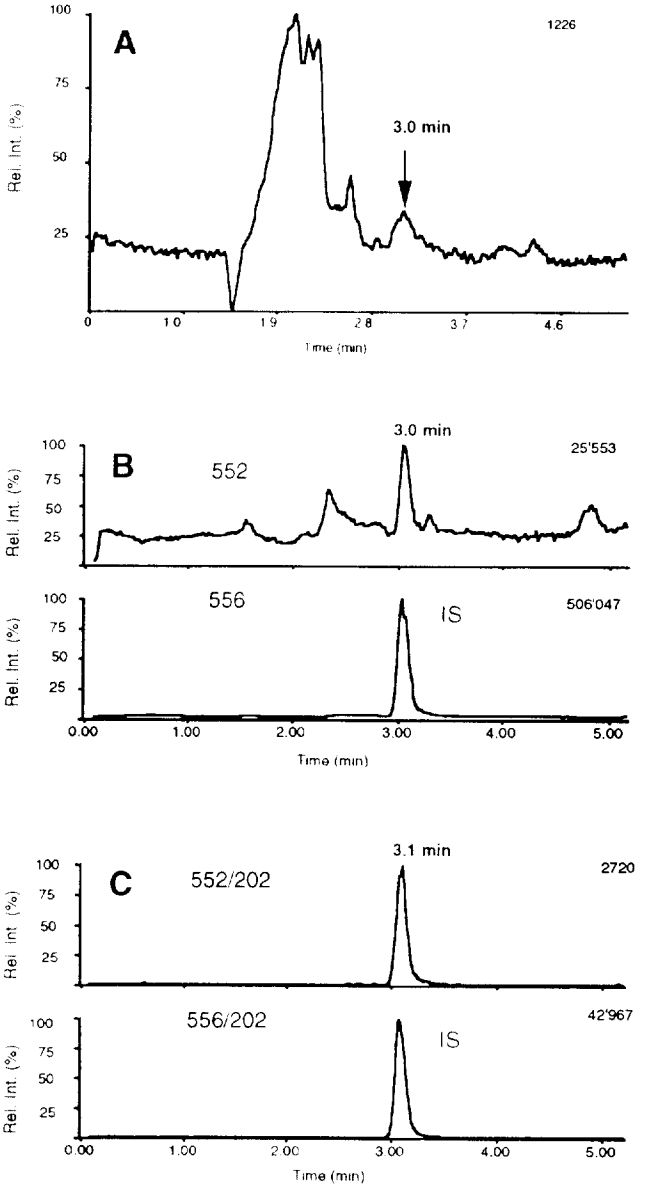


Fig. 2. Analysis of a human plasma extract spiked with 2.5 ng/ml of Ro 47-0203 and 50 ng/ml of Ro 47-0203/d4 by (A) LC–UV, (B) selected-ion monitoring (SIM) and (C) selected reaction monitoring (SRM).

III as I.S.), as illustrated in Fig. 2A, where the analyte and the I.S. are co-eluting with endogenous compounds. Even LC–MS with SIM did not give sufficient selectivity, as shown in Fig. 2B, where the upper trace at m/z 552 represents 2.5 ng/ml of **I**. The use of a gradient instead of an isocratic chromatographic system would certainly help to improve the selectivity, but analysis time would increase considerably. The SRM mode brings the required selectivity to overcome this problem efficiently, as illustrated in Fig. 2C, especially when short retention times are desired. During the analysis of clinical samples, no significant interferences from plasma were observed. To minimize possible interferences from plasma, the resolution of the quadrupoles Q1 and Q3 should not be compromised to improve the sensitivity.

The use of standard-bore columns with the ionspray interface generally requires postcolumn splitting. When biological extracts are analysed, splitting may affect the robustness of the assay. It has been reported that standard ionspray mass spectrometry in the flow-rate range 20–200 μl/min behaves like a concentration-sensitive detector. When using a concentration-sensitive detector, a decrease in the column I.D. from 4.6 to 2 mm will result in a ca. fivefold increase in sensitivity [8]. A further decrease in column diameter to 1 mm or less will result in an increase in sensitivity; however, 2 mm I.D. columns are a good compromise between sensitivity, low solvent consumption and good chromatographic performance. Once the sprayer settings have been optimized for 20 μl/min, an increase in the flow-rate up to 170 μl/min results in a decrease in response of about 40%. When the sprayer position, nebulizer gas flow-rate and interface plate temperature were reoptimized at 170 μl/min, slightly better sensitivity was obtained than at 20 μl/min.

3.2. Internal standard

In the first development phase of the assay, no deuterated internal standard was available. When using short retention times, the choice of the internal standard may be critical because it could interfere with possible metabolites. At this stage, some metabolites were known from in vitro experiments and no interference could be observed from the metabolites or the cyclopropyl analogue used as the IS. Subsequently, a tetradeuterated analogue became available. For mass spectrometry, deuterated internal standards are ideal because they compensate for matrix effects during extraction and matrix suppression during the ionization process. However, pure material is difficult to obtain and often traces of the parent compound are present.

3.3. Assay performance

The precision of the method was evaluated for **I** over the concentration range 0.5–200 ng/ml by replicate analysis of each concentration over a period of several weeks. The data given in Table 1 demonstrate good precision whichever internal standard was used. The assay was linear in the range 0.5–200 ng/ml using a 0.5-ml aliquot of plasma. The recovery from replicate analysis was 87%. A quantification limit of 0.5 ng/ml was found to be sufficient. However, better sensitivity can be achieved, in particular with the new collision cell (API IIIplus). After upgrading the instrument with the new collision cell, the assay was revalidated. The new collision cell allows better mass resolution for Q3 in the MS–MS mode and a fivefold sensitivity increase was obtained. The analyte **I** and the internal standards have different precursor ions, but generate the same product ion; therefore, the setting of the pause time to 50 ms with the new collision cell is very important to avoid carryover.

3.4. Application to biological samples

The method has been applied successfully to the analysis of more than 1500 human plasma samples from clinical trials. Figs. 3 and 4 show representative chromatograms of the analyte with the two different internal standards. In Fig. 4A a peak is observed at a retention time of 3.2 min (280 counts) in the predose trace of the analyte, which corresponds to Ro 47-0203 present at trace levels in the internal standard.

Table 1
Precision and accuracy of the method with the two different internal standards

Amount added (ng/ml)	Ro 47-8761 ($n = 5$)				Ro 47-0203/d4 ($n = 5$)			
	Amount found (ng/ml)	S.D. (ng/ml)	R.S.D. (%)	Inaccuracy (%)	Amount found (ng/ml)	S.D. (ng/ml)	R.S.D. (%)	Inaccuracy (%)
0.50	0.504	0.04	8.91	0.73	0.485	0.03	5.29	3.10
0.75	0.778	0.05	6.03	3.76	0.755	0.05	7.07	−0.71
1.00	0.987	0.04	4.25	1.27	1.014	0.07	6.53	−1.43
2.00	2.190	0.11	5.45	9.52	2.035	0.12	6.03	−1.77
5.00	5.120	0.21	4.17	2.41	5.208	0.14	2.73	−4.17
10.00	10.60	0.22	2.18	6.01	10.27	0.34	3.30	−2.68
50.00	53.40	1.98	3.96	6.79	50.69	0.96	1.89	−1.38
100.00	101.4	2.79	2.79	1.44	103.5	3.59	3.46	−3.55
175.00	173.5	2.67	1.53	0.86	161.9	6.74	4.16	7.46
200.00	193.6	6.75	3.38	3.21	198.5	6.18	3.11	0.74

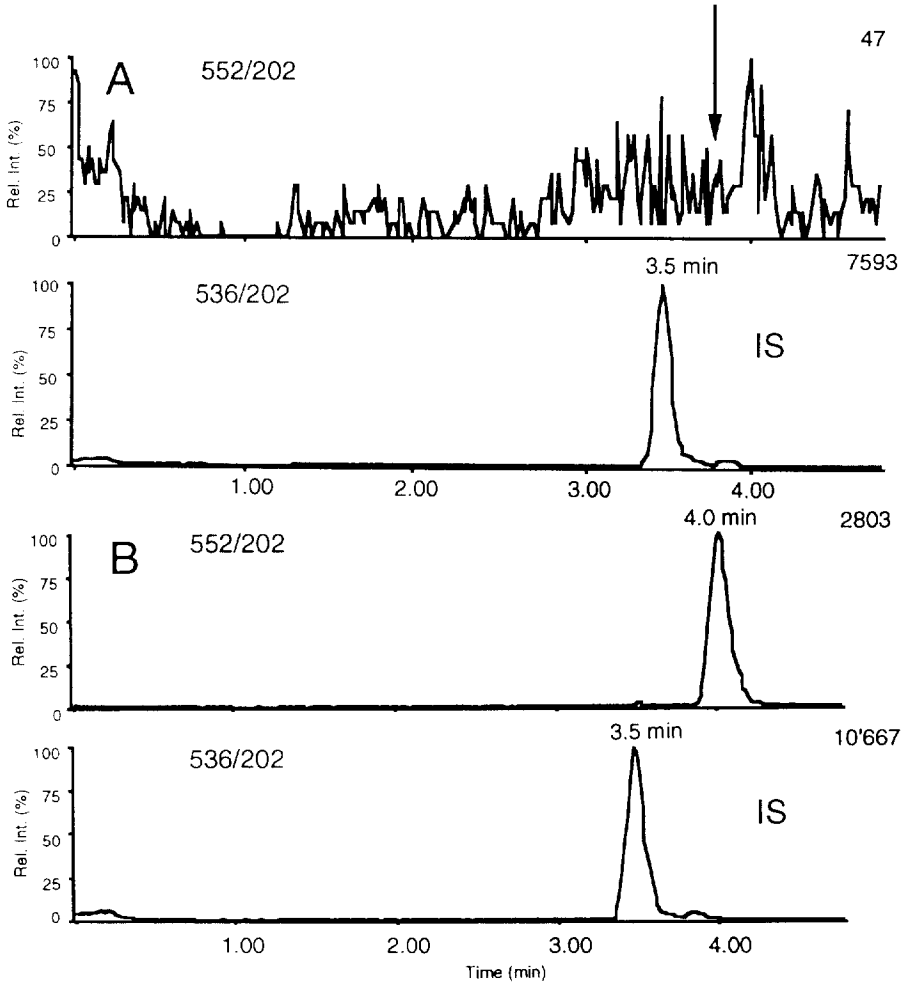


Fig. 3. Selected reaction monitoring (SRM) chromatograms of human plasma with the non-deuterated I.S.: (A) predose and (B) collected after 24 h after a single oral dose of 100 mg p.o. of I: measured concentration, 6.63 ng/ml.

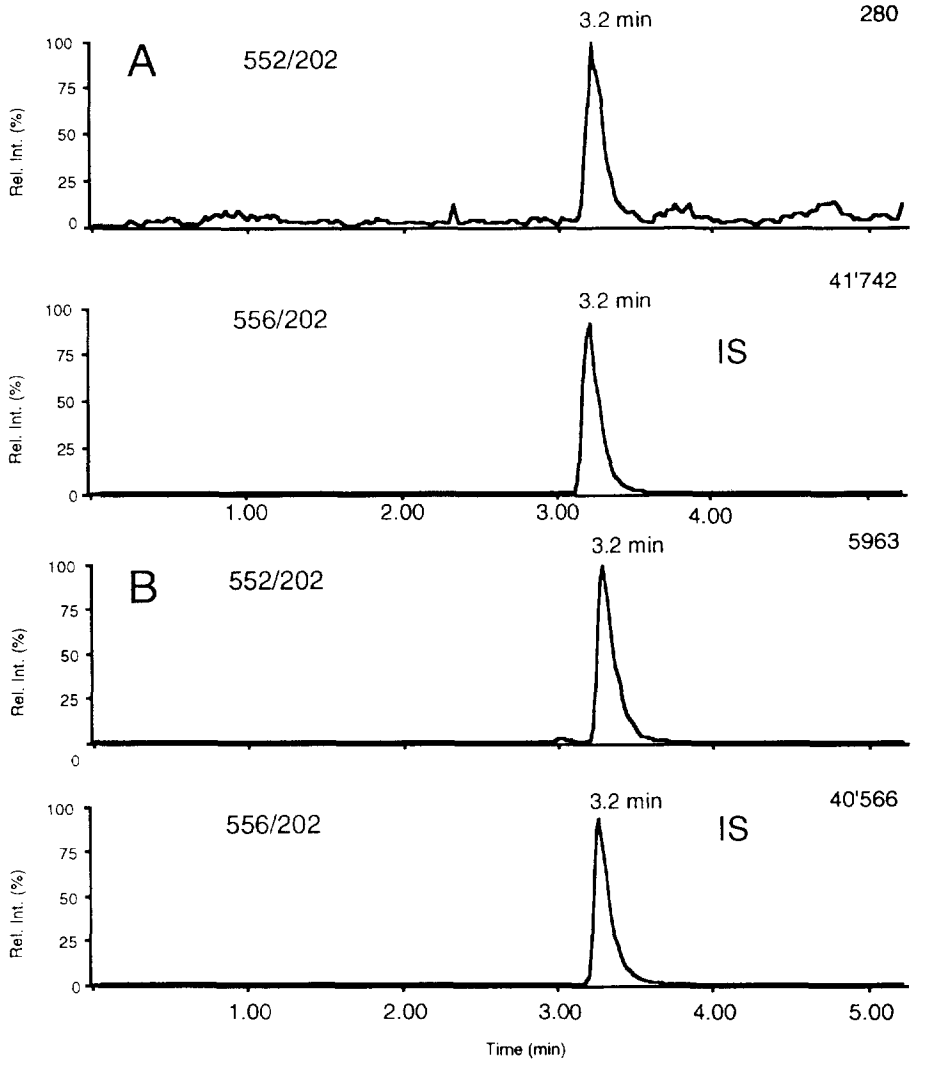


Fig. 4. Selected reaction monitoring (SRM) chromatograms of human plasma with deuterated I.S.: (A) predose and (B) collected after 24 h after a single oral dose of 200 mg p.o. of I; measured concentration, 12.58 ng/ml.

Using MS–MS, the selectivity of the assay was found to be very good and no interfering peaks were observed. The retention time of the analyte was in the range 3–4 min, allowing a short cycle time in the isocratic mode and the analysis of 100 samples can be performed overnight. The narrow-bore columns were found to be reliable and over 500 plasma extracts were injected on to the same column without significant decrease in performance.

Acknowledgement

The authors thank Dr. D. Dell for correcting the manuscript.

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