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Determination of melagatran, a novel, direct thrombin inhibitor, in human plasma and urine by liquid chromatography–mass spectrometry

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Abstract

Analytical methods for the determination of melagatran (H 319/68) in biological samples by liquid chromatography (LC)–positive electrospray ionization mass spectrometry using multiple reaction monitoring are described. Melagatran in plasma was isolated by solid-phase extraction on octylsilica, either in separate extraction tubes or in 96-well plates. Absolute recovery of melagatran from plasma was >92%. Melagatran and the internal standard, H 319/68 D_2 $^{13}C_2$, were separated from other sample components by LC utilizing a C_{18} stationary phase and a mobile phase comprising 35% acetonitrile and 0.08% formic acid in 0.0013 mol/l ammonium acetate solution. After dilution, urine was injected directly onto the LC column and subjected to gradient LC. The relative standard deviation was 1–5% for concentrations above the limit of quantification, which was estimated for plasma at 10 or 25 nmol/l for sample volumes of 500 or 200 μl, respectively, and 100 nmol/l for urine.

Keywords: Melagatran; Thrombin

1. Introduction

Direct thrombin inhibitors represent a relatively new area of advancement within drug research, providing the promise of more effective anticoagulation for the prophylaxis and treatment of a multitude of clinical pathologies.

Melagatran is a direct thrombin inhibitor that can be administered parenterally. It is also the active form of ximelagatran (pINN, formerly H 376/95) a novel, oral direct thrombin inhibitor which is currently undergoing extensive clinical evaluation [1,2]. Ximelagatran is rapidly absorbed and converted to melagatran, the dominant metabolite in plasma, which is eliminated mainly renally in unchanged form (approximately 80%) [3].

As with any new compound undergoing clinical development, a suitable analytical method is required for its determination in biological matrices. Previously, assays for quantification of other thrombin inhibitors in plasma and urine have successfully uti-

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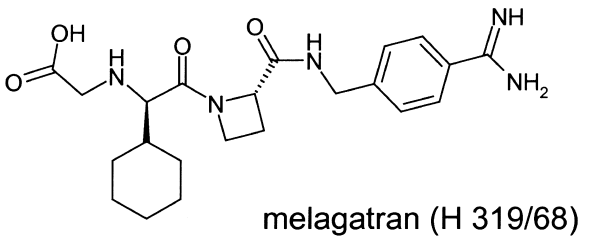
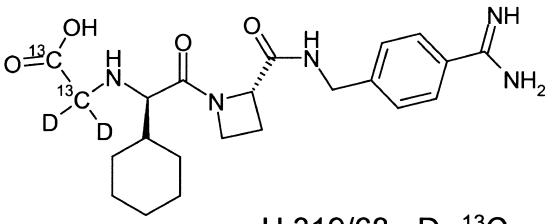
lized liquid chromatography (LC) with fluorescence detection after post-column derivatization [4], UV detection [5,6] or mass spectrometry (MS) [7,8]. Indeed in recent years, tandem MS, which provides superior detection through the elimination of chromatographic interference, has been used for sample analyses in numerous biopharmaceutical studies and for drug therapy monitoring.

This paper describes methods developed for determining the concentration of melagatran in human plasma and urine. Melagatran was isolated from plasma by solid-phase extraction (SPE), separated from co-extractants by reversed-phase LC and measured by positive electrospray ionization MS using multiple reaction monitoring (MRM). An alternate approach for the SPE of melagatran from plasma using 96-well plates is also reported. Melagatran concentrations in urine were determined by LC–MS after a simple dilution procedure.



melagatran (H 319/68)



H 319/68 - D_2 $^{13}C_2$

Fig. 1. Structures of melagatran and the isotope-labelled internal standard, H 319/68 D_2 $^{13}C_2$.

2. Experimental

2.1. Chemicals and materials

Melagatran (H 319/68) and the internal standard H 319/68 D_2 $^{13}C_2$ (Fig. 1) were supplied by AstraZeneca R&D, Mölndal, Sweden. Methanol and acetonitrile of HPLC grade were obtained from Rathburn (Walkerburn, UK). Formic acid, ammonium acetate and hydrochloric acid (Titrisol) were of analytical grade from Merck (Darmstadt, Germany) and high-purity water was obtained from an Elga purification system (High Wycombe, UK). SPE tubes were octylsilica 50 or 100 mg (Varian Bond Elut, Harbor City, CA, USA). The 96-well SPE plates were octylsilica 25 mg (Varian Microlute, Harbor City, CA, USA) or IST Isolute (International Sorbent Technology, Hengoed, UK). A specially designed vacuum tank used at atmospheric pressure that allowed for the direct elution of eluent into micro vials was applied to the extraction tubes. A Rosys Plato 3300 pipetting robot (Quagen Instr., Hombrechticon, Switzerland) or a Tecan RSP150 (Tecan, Hombrechticon, Switzerland) was used for sample extraction in the 96-well plates.

2.2. Blood collection

Blood from the antecubital vein was placed in polypropylene tubes containing anticoagulant. Routinely, heparin is the anticoagulant used in blood sample collection for drug analysis. However, in some studies where the anticoagulant activity of melagatran was being simultaneously evaluated, blood samples were collected in citrated tubes. No change in sample preparation was required for citrated plasma. Blood samples were spun for 10 min at 1500 *g* enabling separation of plasma. The plasma was then transferred to polypropylene cryo vials and stored frozen at −20°C until analysis.

2.3. Liquid chromatography–mass spectrometry

Chromatographic experiments were performed using a Perkin-Elmer 200 series (Uberlingen, Germany) LC pump and autosampler. LC separations were undertaken at ambient temperature (21–23°C). A reversed-phase Hypersil BDS-C_{18} 5 μm analytical column (plasma: 100 mm×4.0 mm I.D.; urine: 100

mm×3.0 mm I.D.) obtained from ThermoQuest (Runcorn, UK) or a similar column was employed. For melagatran plasma sample analysis, isocratic elution was employed using a mobile phase containing 35% acetonitrile and 0.08% formic acid in 0.0013 mol/l ammonium acetate solution. This gave a retention time of approximately 1.5 min with a flow-rate of 0.75 ml/min.

For determination of melagatran in urine samples, mobile phase constituents were the same as those used for plasma samples (acetonitrile and 0.08% formic acid in 0.0013 mol/l ammonium acetate solution), but a step gradient programme with 10% and 60% acetonitrile solutions at a flow-rate of 0.75 ml/min was used. The acetonitrile content was 20% for 0.70 min, thereafter 60% until 2.00 min, 12.5% until 3.00 min, and returned to 20% 0.10 min before injection of next sample. This system provided a retention time of approximately 1.0 min.

The mass spectrometer was a Perkin-Elmer Sciex API 3+, API 365, API 2000 or API 3000 triple quadrupole with electrospray interface (Concord, Canada). The effluent from the LC column was split in a Valco-tee connection (Valco International, Schenkon, Switzerland) in order that liquid flow to the electrospray interface was 30 μl/min for API 3+ and 100–200 μl/min for API 365, API 2000 and API 3000, which were equipped with turbo-ion spray. Typical settings for orifice voltage was 40–55 V and for collision energy (Q0-RO2) 23 eV (API 3+), 27 eV (API 365), 31.5 eV (API2000) and 34 eV (API3000). Other settings, including gas flow, lens and quadrupole voltages and parameters for mass resolution of the separating quadrupole were used as obtained during routine optimization of the instrument. Mass spectral peak width ($w_{1/2}$) was typically 0.7–0.8 u for Q1 and 1.0–1.2 u for Q3 in MRM measurements.

MRM transitions of the protonated molecular ions were used for quantitative analysis, i.e., m/z 430.2→233.0 for melagatran and m/z 434.2→233.0 for the internal standard H 319/68 D_2 $^{13}C_2$. A dwell time of 200 ms on each ion transition was used for the 4-mm I.D. column and 100 ms for faster separations on the 3-mm I.D. column. Chromatograms were smoothed (smoothing factor 2) before peak integration.

Statistical evaluations of between-day and within-day repeatability were undertaken using analysis of variance (ANOVA) [9].

2.4. Solid-phase extraction of plasma samples

Melagatran (glycine, *N*-[(1*R*)-2-[(2*S*)-2-{[([4-(aminoiminomethyl)phenyl]methyl)amino]carbonyl}-1-azetidinyl]-1-cyclohexyl-2-oxoethyl]) (Fig. 1) is a peptidomimetic compound with protolytic functions, one carboxylic acid and two amino groups, with pK_a values of 2, 7 and 11.5. The distribution ratio between 1-octanol and carbonate buffer at pH 9.7 is approximately 0.05 or log $D=-1.33$. SPE was chosen as an appropriate method to isolate melagatran from human plasma.

2.4.1. Extraction tubes

Extraction of melagatran from plasma was undertaken in purpose-made extraction tanks. The SPE tubes were placed in drill holes in the lid of the tank. A stainless steel needle was attached to the lid under each hole, in order to increase the gravitational flow through the sorbent (cf. 96-well plate format below). Thawed plasma samples were mixed and centrifuged for 5 min before extraction. SPE tubes (50 or 100 mg tubes for 200 and 500 μl plasma, respectively) were activated by the addition of 1 ml methanol and conditioned with 1 ml of acetonitrile–water (50:50) followed by 1 ml 0.1% aqueous formic acid solution. A 50-μl volume of the labelled melagatran internal standard solution, 50 μl of standard solution (for plasma standards only) and 200 or 500 μl of plasma sample or blank plasma (for plasma standards only) were added to each tube and drawn through by gravity. Residual plasma was washed off with 1 ml aqueous 2 mmol/l ammonium acetate solution before elution of melagatran with 1 ml acetonitrile–2 mmol/l ammonium acetate (35:65). The eluent was collected in micro vials and centrifuged for 5 min before injection of 10 (30) μl onto the LC column.

2.4.2. 96-Well plates

In order to enhance the gravitational flow in the 96-well plates with low-mass sorbent extraction beds, a spigot extension plate outfitted with one stainless steel needle for each well was attached to

the bottom of the 96-well plate. The liquid column formed by the use of the spigot extension plate increased the flow through the extraction bed in the 96-well plate. Due to capillary forces and surface tension, the flow stopped as soon as the fluid above the extraction bed was consumed.

Each SPE well was activated by the addition of 500 μl methanol and conditioned with 250 μl acetonitrile–water (50:50) followed by 250 μl 0.1% aqueous formic acid solution. A 50-μl volume of internal standard solution and 200 μl of plasma sample or a premixed standard plasma sample were added to each extraction well, and eluted by gravity. The SPE wells were washed with 500 μl 2 mmol/l ammonium acetate before elution with 500 μl acetonitrile–2 mmol/l ammonium acetate (35:65). The eluent was collected in a deep-well plate that was sealed, mixed and centrifuged for 5 min prior to injection of 10 (30) μl onto the LC column.

2.5. Preparation of urine samples

Thawed urine samples were mixed and centrifuged for 5 min. A dilution solution containing the internal standard (400 nmol/l) was made from 900 ml ammonium acetate (4 mmol/l), 100 ml acetonitrile and 1.0 ml formic acid, whereafter 1000-μl volumes were transferred to injection vials. A 100-μl aliquot of urine was then added to each vial, mixed and centrifuged for 5 min. A 3-μl volume of this diluted urine sample was then injected onto the LC column. Urine standards, prepared by adding melagatran to blank urine, were treated as patient samples.

3. Results and discussion

3.1. Extraction of melagatran from plasma

SPE was used to isolate melagatran from plasma. For practical purposes the internal standard was added to the extraction tubes/96-well plate prior to the addition of the plasma sample. This approach was found to make no difference relative to that of premixing the internal standard and plasma prior to SPE. For both approaches, an absolute recovery of more than 92% was obtained for melagatran and the internal standard. Assessment of the recovery of melagatran was estimated by comparing the LC–MS results from plasma samples where melagatran was added prior to SPE, with those where melagatran (in 50 μl, 0.010 mol/l HCl) was added to plasma extracts obtained after SPE. HCl (50 μl, 0.010 mol/l) was also added to the processed sample extract. These precautions were taken to avoid MS detector response variation caused by sample matrix differences.

Melagatran recovery was found to be highly affected by the sample flow-rate during SPE, both for the extraction tubes with 50–100 mg solid-phase material and for the 96-well plates with low-mass sorbent extraction beds. High flow-rates by applied vacuum gave, as in other SPE applications, worse recovery and repeatability. Hence, only gravity, without an external vacuum source (see Experimental), was used to drive the flow during extraction.

3.2. Liquid chromatographic separation

3.2.1. Plasma

Ion chromatograms for melagatran and the internal standard of a plasma sample from a patient are shown in Fig. 2. The corresponding chromatogram from a blank plasma sample (overlaid in Fig. 2A) shows a small contribution from unlabelled melagatran present in the internal standard. The sample turnover was 20 injections/h.

Only a few reversed-phase materials gave satisfactory retention of melagatran, and batch variation was observed. Column performance is important, since comigration of melagatran and early eluting components from the plasma extract can result in variation of response suppression. Analyte retention was therefore controlled daily by LC–MS via a window scan of 2 u around the precursor ions. A typical total ion trace of such an analysis is shown in Fig. 3. If required for adequate separation, the retention of melagatran (m/z 430.2, t_R 1.3 min) was adjusted by modification of the mobile phase. The first peak (t_R 1.15 min) at the void volume emanated from m/z 430.8 (Fig. 3B). A single-MS spectrum of the first peak (not displayed) showed a series of ions, presumably of adducts formed by sodium and formate ions. The ion detected at m/z 430.8 is assumed to represent $[(Na^+)_{n+1}(HCOO^-)_n]^+$, where $n=7$.

The suppression of the signal from a plasma

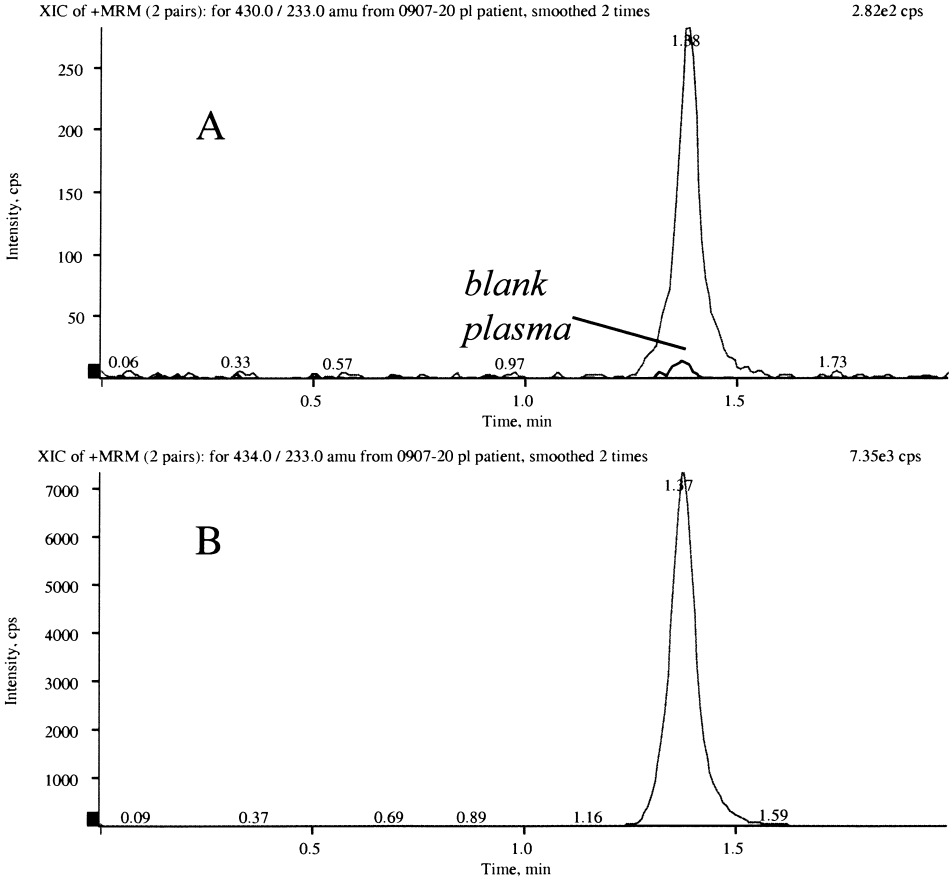


Fig. 2. LC–MS analysis of a patient plasma sample (500 μl) containing (A) 17 nmol/l melagatran and (B) 0.7 μmol/l internal standard. The ion chromatogram for melagatran from a blank plasma sample is overlaid in (A). Sample preparation: 100 mg SPE tubes. Conditions: LC column C_{18}, 5 μm, 100 mm×4 mm I.D., injection volume 30 μl, flow-rate 0.75 ml/min. MS instrument: API 3^+. Dwell time: 200 ms.

extract amounts to about 50% relative to pure aqueous solution as experienced on different MS instruments by us and by other laboratories setting up the method.

Initially, good column stability was obtained by injecting 30 μl of eluent from the SPE of plasma on to the LC column. However, later batches of packing material showed reduced column stability leading to peak distortion and retention drop. A reduced injection volume (10 μl) was found to provide very good column durability and is used for LC–MS analysis on the modern sensitive API 3000 instrument. When still injecting 30 μl volumes, the column stability was improved by pH adjustment through the addition of 10 μl of 10% formic acid to 1 ml of extract, which thereby conformed to the mobile phase constituents.

3.2.2. Urine

A step gradient of acetonitrile in the mobile phase was introduced for the determination of melagatran in urine to avoid interference from components eluting before melagatran and also to clean the LC column from late eluting impurities emanating from directly injected, diluted urine samples. Fig. 4 shows ion chromatograms of a urine sample from a patient using the 3-mm I.D. column at increased linear flow-rate (volume flow-rate was the same as that used for plasma samples, 0.75 ml/min). An expanded melagatran trace from a blank urine sample

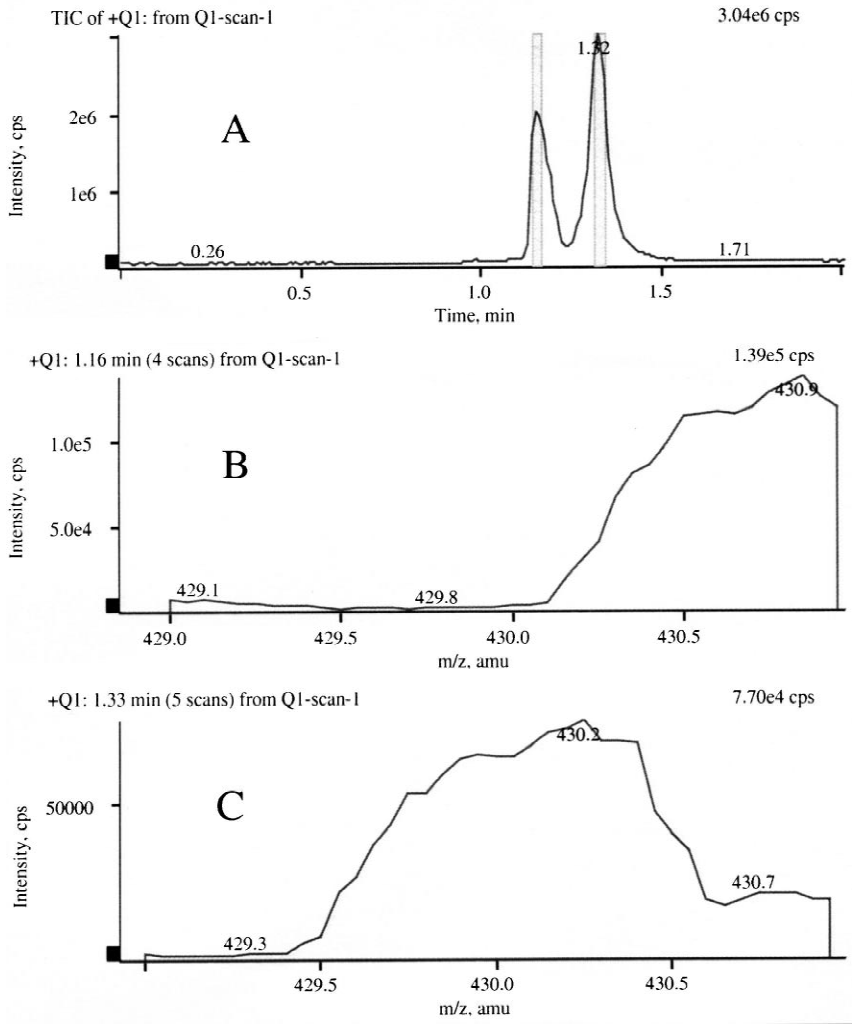


Fig. 3. Single MS scan for control of separation quality. Plasma sample extract, 0.6 μmol/l melagatran and 0.7 μmol/l internal standard. (A) Total ion chromatogram, (B) MS scan from the first peak, void and (C) MS scan from the second peak, melagatran. MS instrument: API 3^{+}. Scan range 429–431 and 433–435 u, step 0.05 u, dwell time 6 ms. Orifice voltage 40 V.

is shown in Fig. 4B, showing that chromatographic interference is largely eliminated by LC–MRM analysis. The sample turnover was 16 injections/h.

In this method the use of the API 3000 instrument, which maintains sensitivity at short dwell times, is advantageous because it allows for sensitive detection of fast eluting peaks, which occur as a result of increased linear flow-rate. In addition, a small volume of diluted urine (3 μl) can be injected on to the LC column, thus reducing ion source contamination.

3.3. MS detection

Product ion spectra from collision induced dissociation of the protonated molecular ions of melagatran and the internal standard are shown in Fig. 5.

The most abundant product ion at m/z 233.0 was used for MRM for both molecular species. With identical product ions there is a risk of ''crosstalk'' between the signals from the two MRM transitions due to slow migration of ions through the collision

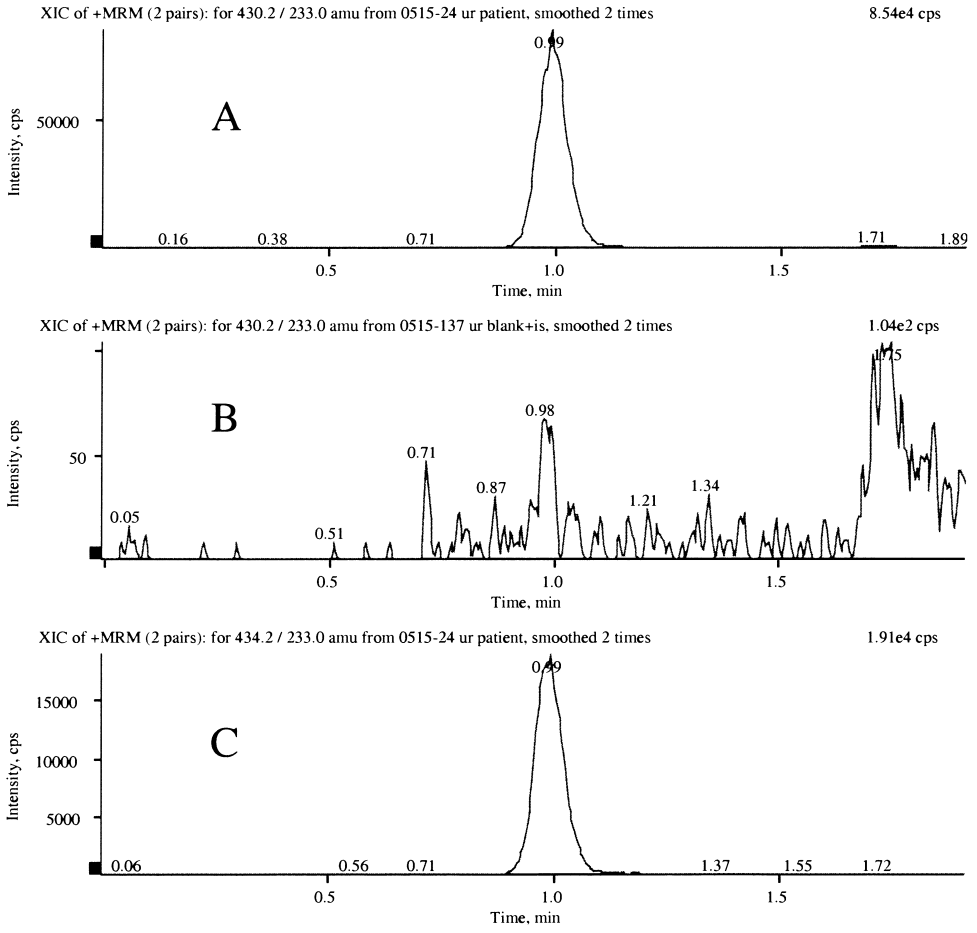


Fig. 4. LC–MS analysis of a patient urine sample containing (A) 10.1 μmol/l melagatran and (C) 4 μmol/l internal standard. An expanded trace from a blank urine sample is shown in (B) (internal standard not displayed). Conditions: LC column C_{18}, 5 μm, 100 mm×3 mm I.D., injection volume 3 μl, flow-rate 0.75 ml/min, step gradient elution between 0.7 and 1.7 min. MS instrument: API 3000. Dwell time: 100 ms, pause time 5 ms.

cells of the API 3+ and API 365 instruments. Such crosstalk may be observed both as a false background of melagatran in blanks containing the internal standard, and as increased internal standard response for samples with very high melagatran concentrations. The latest versions of the software for API 365 (MassChrom 1.1) and API 3+ (API Software 2.6 [FPU]) addressed this problem by use of a settling function, which emptied the collision cell between MRM transitions. In the API 2000/3000 instruments, the use of a linear accelerating potential along the collision cell eliminated measurable crosstalk.

3.4. Stability

Melagatran in 0.01 *M* HCl was stable for at least 13 months stored at 4–8°C. No degradation was seen in heparinised or citrated plasma samples stored at −20°C after 12 months or at room temperature after 2 days. Six repeated freezing–thawing cycles did not influence the original concentration of melagatran. In processed plasma samples stored at room temperature, quantitative results from day 2 agreed well with those from day 0. A similar finding was observed for processed samples stored for 2 months at 4–8°C with airtight, non-punctured vial caps.

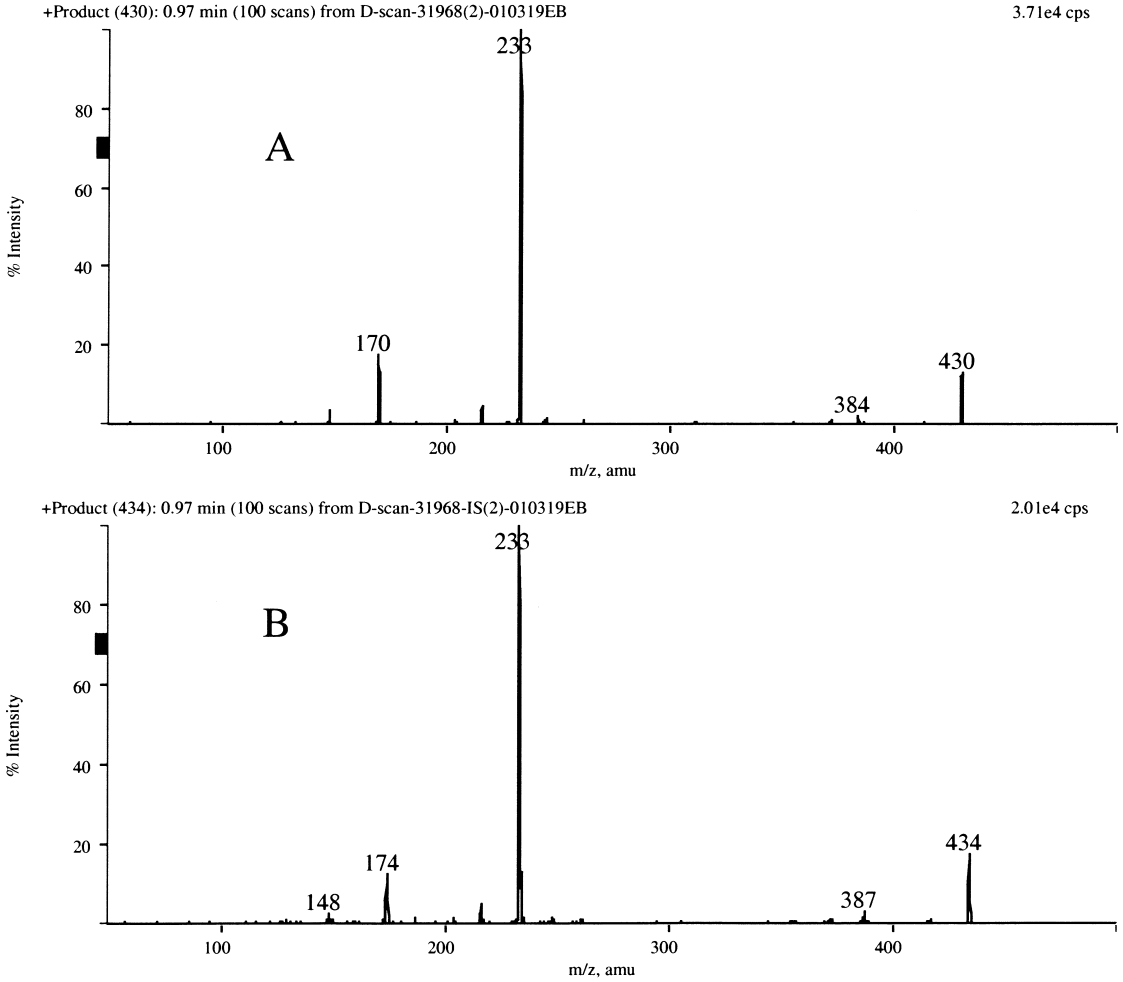


Fig. 5. Product ion spectra for (A) melagatran and (B) internal standard, H 319/68 D_2 $^{13}C_2$. Precursor ions: protonated molecular ions at *m/z* 430.2 and 434.2, respectively. The most abundant product ion at 233.0 was used for MRM. Conditions: infusion of 1.6 μmol/l melagatran and internal standard in mobile phase at 10 μl/min, mixed with mobile phase 100 μl/min in a Valco-tee and transferred to the turbo-ion spray. MS instrument: API 3000, dwell time 0.25 ms, step 0.1 u.

In urine, melagatran showed stability for at least 5 months when stored at −20°C and remained stable after four freeze–thaw cycles. There was no decline in concentration after 24 h at room temperature or in processed samples after 8 days at 4–8°C.

3.5. Linearity and calibration

Standard curves consisting of eight plasma concentrations of melagatran (0.010–10 μmol/l) performed in triplicate showed linearity (inaccuracy< 15%). Repeatability was estimated at four concentrations, 25, 50, 1000 and 10000 nmol/l. The relative standard deviation (RSD) was approximately 3% at 25 nmol/l (200 μl plasma) and between 1 and 2% at higher melagatran concentrations. The limits of quantification (LOQs) were approximately 10 and 25 nmol/l for 500 and 200 μl of plasma (RSD<20%), respectively, for the extraction tube procedure, and 10 nmol/l for 200 μl plasma for the 96-well procedure.

Reproducibility or between-day repeatability was

estimated from quality control plasma samples that were analysed along with patient samples on 248 occasions. The average was 98.5% of the nominal melagatran plasma concentration (1 μmol/l), with an RSD of 3.0%. Daily calibration of the analytical method was performed using 10 and 600 nmol/l standard plasma melagatran concentrations ($n=6$). Quality control samples were routinely analysed daily at a concentration of approximately 300 nmol/l ($n=2–4$). Full standard curves were run intermittently or every second week to confirm linearity.

For analysis of melagatran concentrations in urine, the LOQ was estimated at 100 nmol/l with linearity up to 280 μmol/l. Repeatability was satisfactory with an RSD of less than 5% at concentrations above the LOQ. Urine standards at 100 nmol/l and 6 μmol/l were used for daily calibration of the analytical method.

4. Conclusions

In the present study, we demonstrated that a simple SPE method was suitable for the extraction of melagatran, a direct thrombin inhibitor, from human plasma using extraction tubes. A 96-well extraction plate allowed for automation of the sample preparation step. The LC–MRM-MS analysis provided high selectivity, good precision and accuracy, and short analysis times for both plasma and urine samples.

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