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Determination of ximelagatran, an oral direct thrombin inhibitor, its active metabolite melagatran, and the intermediate metabolites, in biological samples by liquid chromatography–mass spectrometry

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Abstract

Analytical methods for the determination of ximelagatran, an oral direct thrombin inhibitor, its active metabolite melagatran, and intermediate metabolites, melagatran hydroxyamidine and melagatran ethyl ester, in biological samples by liquid chromatography (LC) positive electrospray ionization mass spectrometry (MS) using selected reaction monitoring are described. Isolation from human plasma was achieved by solid-phase extraction on octylsilica. Analytes and isotope-labelled internal standards were separated by LC utilising a C_{18} analytical column and a mobile phase comprising acetonitrile–4 mmol/l ammonium acetate (35:65, v/v) containing 0.1% formic acid, at a flow-rate of 0.75 ml/min. Absolute recovery was ~80% for ximelagatran, ~60% for melagatran ethyl ester and >90% for melagatran and melagatran hydroxyamidine. Limit of quantification was 10 nmol/l, with a relative standard deviation <20% for each analyte and <5% above 100 nmol/l. Procedures for determination of these analytes in human urine and breast milk, plus whole blood from rat and mouse are also described.

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1. Introduction

The search for antithrombotic drugs that are safe and effective and can be administered orally is an area of high priority in cardiovascular drug research. Ximelagatran is a new oral direct thrombin inhibitor [1–3], which is currently undergoing extensive clinical evaluation as an anticoagulant and antithrombotic agent in prophylaxis and treatment of various thromboembolic conditions [4–7]. Ximelagatran is rapidly absorbed and converted to melagatran, the active form and major metabolite in plasma [2,8,9]. In order to allow for quantitative drug analysis to support preclinical and clinical evaluation, methods for determination of ximelagatran, melagatran, and two intermediate metabolites in biological matrices are required.

Liquid chromatography (LC) with various methods of detection [10–14] has been shown to be

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successful for the quantification of thrombin inhibitors in plasma and urine. Indeed, recently we presented an analytical method for the determination of melagatran in human plasma and urine using LC–MS with positive electrospray ionization [15]. As an extension to this work, this paper describes a method for the determination, from various biological matrices (including human plasma, urine and breast milk, plus whole blood from rats and mice), of ximelagatran, its major metabolite and active form melagatran, plus two intermediate metabolites, melagatran hydroxyamidine (OH-melagatran) formed by hydrolysis of the ethyl ester, and melagatran ethyl ester (ethyl-melagatran), formed by the reduction of the hydroxyamidine group in ximelagatran.

2. Experimental

2.1. Chemicals and materials

Ximelagatran, OH-melagatran, ethyl-melagatran, melagatran and their respective deuterium (D) and ^{13}C-labelled internal standards (Fig. 1A and B) were supplied by AstraZeneca R&D, Mölndal, Sweden. HPLC grade methanol and acetonitrile were purchased from Rathburn (Walkerburn, UK) and 1-octanol from Riedel–de-Haën (Seelze, Germany). Analytical grade formic acid, ammonium acetate and hydrochloric acid (HCl [Titrisol]) were purchased from Merck (Darmstadt, Germany). High purity water was obtained from an Elga purification system (High Wycombe, UK). Standard solutions of the analytes and their internal standards were prepared in 0.01 mol/l HCl. Solid-phase extraction (SPE) tubes were either octylsilica 100 mg (Varian Bond Elut, Harbor City, CA, USA) or OASIS HLB (Waters, Milford, MA, USA). Sodium dodecylsulfate (SDS) was purchased from Sigma (St. Louis, MO, USA).

2.2. Blood collection

Blood from the antecubital vein was placed in polypropylene tubes containing anticoagulant. Heparin is the anticoagulant used routinely in blood sample collection for drug analysis. However, in studies where anticoagulant activity was also being determined, blood samples were collected into citrated (trisodium citrate) tubes. No change in sample preparation was required for citrated plasma.

Blood samples were spun within 1 h of sampling for 10 min at 1500 *g*, to separate plasma. The plasma was then transferred to polypropylene cryo-vials and stored frozen at −20 °C until analysis.

2.3. Liquid chromatography–mass spectrometry

The liquid chromatographic system consisted of a Perkin-Elmer 200 series autosampler and LC pump (Überlingen, Germany), or an HTS PAL injector (CTC Analytics, Zwingen, Switzerland) combined with an HP 1100 series LC pump (Agilent Technologies Deutschland, Waldbronn, Germany). LC separations were undertaken at room temperature (21–23 °C) on a reversed-phase Hypersil BDS-C_{18} 5 μm analytical column (100×4.0 mm I.D.) obtained from ThermoQuest (Runcorn, UK) or a similar column. The mobile phase used for plasma samples was acetonitrile–4 mmol/l ammonium acetate (35:65, v/v) containing 0.1% formic acid, giving an apparent pH of about 3.3. The analytes co-eluted with their respective isotope-labelled internal standards. In the gradient elution systems used for other matrices, the final concentration of formic acid was always 0.1%, while the proportion between acetonitrile and ammonium acetate buffer was varied.

The effluent from the LC column (flow-rate 0.75 ml/min) was split with a Valco T-connection (Valco International, Schenkon, Switzerland), giving a liquid flow of 100–200 μl/min to the mass spectrometer. The MS was a Perkin-Elmer Sciex API 3000 or API 365 triple quadrupole with electrospray (turbo-ion spray) interface (Concord, Canada). Typical settings were 45 V for the orifice voltage, 30–34 eV for collision energy, collision-activated dissociation gas at 3 and turbo heater temperature at 375 °C. Mass spectral peak width ($w_{1/2}$) was typically 0.7–0.8 u for Q1 and 1.0–1.2 u for Q3 for the selected reaction monitoring (SRM) measurements for API 365, while unit mass resolution was used for the API 3000 instrument due to its higher sensitivity. The SRM transitions of the precursor ions $(M+H)^+$ and the corresponding product ions are shown in Table 1. Product ions were monitored in two time periods to allow for a longer dwell time. A dwell time of 200 ms was used for all four analytes with the API 3000,

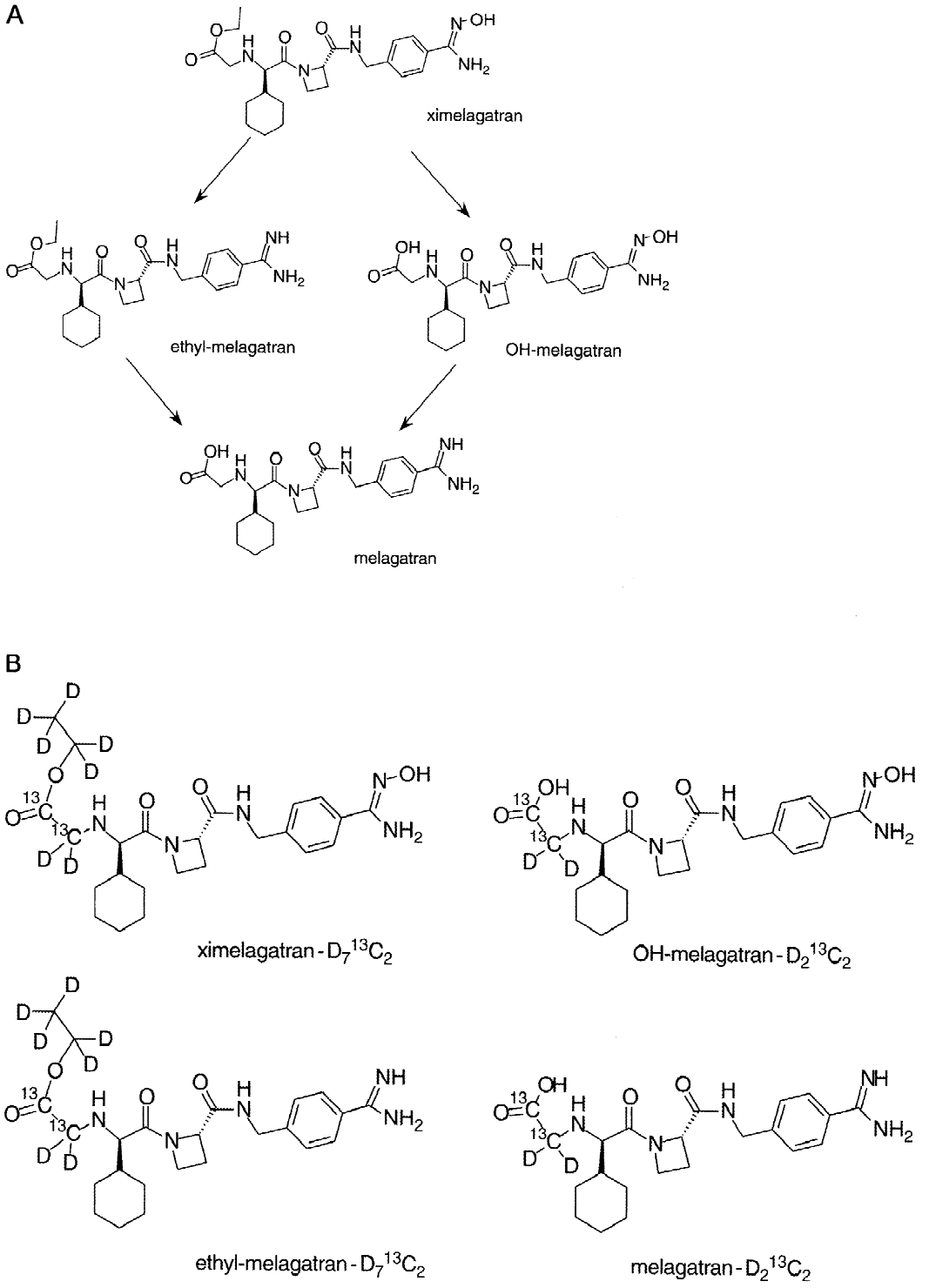


Fig. 1. (A) Structures of ximelagatran, intermediate metabolites OH-melagatran and ethyl-melagatran, and melagatran, and (B) the corresponding isotope-labelled internal standards.

Table 1
Selected reaction monitoring (SRM) transitions used for quantification

	Precursor ion m/z $(M+H)^+$	Product ion m/z
Melagatran	430	233
Melagatran-$D_2^{13}C_2$	434	233
OH-melagatran	446	249
OH-melagatran-$D_2^{13}C_2$	450	249
Ethyl-melagatran	458	233
Ethyl-melagatran-$D_7^{13}C_2$	467	233
Ximelagatran	474	198
Ximelagatran-$D_7^{13}C_2$	483	207

while for the API 365, 400 ms was used for OH-melagatran, and 200 ms for the three other analytes. A dwell time of 100 ms was used for the internal standards on both instruments.

2.4. Solid-phase extraction of human plasma samples

2.4.1. Rationale for use of solid-phase extraction

Ximelagatran, ethyl [((1*R*)-2-{(2*S*)-2-[({4-[amino-(hydroxyimino) methyl] benzyl} amino) carbonyl] azetidin - 1 - yl} - 1 - cyclohexyl - 2 - oxoethyl)amino]acetate, has pK_a values of 5.2 (hydoxyamidine) and 4.5 (secondary amine) [2]. Melagatran, [((1*R*)-2-{(2*S*)-2-[({4 - [amino(imino)methyl]benzyl}amino)carbonyl]-azetidin - 1 - yl} - 1 - cyclohexyl - 2 - oxoethyl)amino]-acetic acid, has pK_a values of 2.0, 7.0 and 11.5 [2]. Melagatran has a distribution ratio to 1-octanol of approximately 0.05 at pH 9.7, where the compound is net neutral, i.e. $\log K_D = -1.3$, while ximelagatran is much more lipophilic and $\log K_D = 0.9$ (at pH 7.0) [2]. Intermediate metabolites are OH-melagatran, [((1*R*)-2-{(2*S*)-2-[({4-[amino(hydroxyimino)methyl]-benzyl} amino) carbonyl]azetidin-1-yl}-1-cyclohexyl-2-oxoethyl)amino]acetic acid, and ethyl-melagatran, ethyl [((1*R*) - 2 - {(2*S*) - 2 - [({4 - [amino(imino)methyl]-benzyl}amino)carbonyl]azetidin - 1 - yl} - 1 - cyclohexyl-2-oxoethyl)amino]acetate.

The different protolytic properties of the four compounds and the hydrophilic character of melagatran made liquid–liquid extraction difficult why SPE was chosen as the isolation method from human plasma.

2.4.2. Solid-phase extraction procedure

Thawed plasma samples were mixed and then centrifuged at 2500 *g* for 15 min at 20 °C just prior to extraction. For SPE, purpose-made extraction tanks, with the SPE tubes placed in drill holes in the lid of the tank, were used as described previously [15]. A stainless steel needle was attached to the lid under each hole to promote gravitational flow through the sorbent. The Bond Elut SPE tubes were activated by the addition of 1 ml methanol and conditioned with 1 ml 0.1% formic acid solution. A 50-μl volume of the working internal standard solution (containing approximately 4000 nmol/l of each internal standard), 50 μl of working standard solution (containing 4000 or 100 nmol/l of each analyte) for high and low plasma concentration standards (six replicates of each) and 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 of 2 mmol/l ammonium acetate solution before elution with 1 ml of elution liquid, consisting of 4% ammonia solution (25% aq.) in methanol. The eluent was collected in glass tubes and the solvent evaporated under nitrogen at 30 °C. The extract was reconstituted in 500 μl acetonitrile–4 mmol/l ammonium acetate (20:80, v/v) containing 0.4% formic acid, and the redissolution liquid was vortex-mixed for 2 min before being transferred to autosampler vials. A 200-μl volume of 1-octanol was added to each vial, which was then vortex-mixed for 2 min and centrifuged at 2500 *g* for 5 min at 20 °C. The vials were placed in the autosampler, and 10 μl of the lower phase were injected onto the LC column.

2.5. Preparation of human urine samples

Thawed urine samples were mixed and then centrifuged at 2500 *g* for 5 min at 20 °C. A 100-μl urine sample was then transferred to a 1.5-ml injection vial containing 1000 μl acetonitrile–4 mmol/l ammonium acetate (10:90, v/v), containing 0.1% formic acid. After addition of 50 μl working internal standard solution and mixing, 30 μl was

injected onto the LC column. Elution was carried out in a linear gradient by raising the acetonitrile content of the ammonium acetate–formic acid buffer solution from 20 to 60%.

2.6. Preparation of human breast milk samples

Thawed breast milk samples were gently mixed. Excess fat in the sample was removed step-wise by repeated centrifugation, separation and transfer to a new vial prior to analysis. The first centrifugation was performed at 4300 *g* for 10 min at 10 °C, and the second at 22 000 *g* for 15 min at 5 °C. SPE of a 1-ml sample was carried out according to the method for plasma described above, but omitting the final clean-up extraction with 1-octanol. An acetonitrile step gradient from 10 to 60% in ammonium acetate–formic acid buffer was applied for the LC separation in this assay.

2.7. Stabilisation and SPE of whole blood samples from rat and mouse

Esterase activity is significantly higher in rat and mouse blood than in human, resulting in rapid degradation of ximelagatran and ethyl-melagatran in these samples. In order to assay these two compounds more accurately in these species and obtain more representative data, whole blood was collected in sample tubes containing an equal volume of 10% SDS solution and mixed immediately to denature proteins and thus inhibit analyte degradation. The samples were further stabilised by storage at −70 °C (see Ref. [16]). Two hundred μl of this blood mixture, 50 μl 0.01 mol/l HCl, 50 μl working internal standard solution and 750 μl water were mixed and centrifuged at 2500 *g* for 5 min at 20 °C, prior to isolation on OASIS HLB SPE columns. The washing step was also extended, relative to that used in the plasma procedure, by 2 ml of water and 2 ml of 40% methanol, each in 1-ml portions, prior to elution. Whole blood standards were prepared by adding the working standard solution to whole blood premixed with SDS solution and were then run in parallel to the authentic samples. LC separation used a similar mobile phase as described above, the only modification being the addition of an acetonitrile step gradient of 20, 40, and 60%. (A detailed procedure for this assay is available on request). Quantitative analysis of OH-melagatran was not performed with this method.

3. Results and discussion

3.1. Solid-phase extraction of plasma samples

Ximelagatran, melagatran and the two intermediate metabolites (OH-melagatran and ethyl-melagatran, Fig. 1A) were isolated from plasma using SPE. With manual multipipettes or even more for robotic pipetting, addition of the working standard and internal standard solutions directly to the extraction tubes instead of premixing with the plasma sample facilitates the procedure significantly. In comparative studies this was not found to influence the accuracy of the analysis relative to premixing prior to SPE. Absolute recoveries were determined by comparison with standards spiked to blank extracts and were approximately 80% for ximelagatran, 60% for ethyl-melagatran, and >90% for melagatran and OH-melagatran. Recoveries were of the same magnitude for the respective isotope-labelled internal standards (Fig. 1B). Gravitational flow through the SPE columns was found to be preferential in terms of precision and accuracy, compared with forced flow applied by vacuum or pressure. The low absolute recovery for ximelagatran, and the even lower recovery for ethyl-melagatran, was found to be fully compensated for by the internal standards.

As might be anticipated, the four analytes, which differ in protolytic groups and lipophilicity, exhibited varying degrees of retention on the silica-based reversed-phase material used for SPE. In order to avoid loss of melagatran and OH-melagatran, plasma residues were washed out of the column with aqueous buffer without any organic modifier. A combination of ammonium hydroxide and a high concentration of methanol was needed for adequate recovery of ethyl-melagatran, whilst, as found previously [15] an ammonium acetate buffer with a moderate concentration of acetonitrile was found to be sufficient for efficient recovery of melagatran. The fact that ethyl-melagatran was more strongly

retained than ximelagatran suggests that ion-exchange interactions with silanol groups may play an important role in retention.

During the development of this analytical method it was found that when undertaking LC separation, late-eluting components from the previous injection could cause significant signal suppression. One way to avoid this would be to introduce a step gradient at the end of each run, however, this would have the disadvantage of doubling the time between injections. An alternative method was to modify the pre-treatment of the sample, as it was found that the interfering coextractants could be removed by liquid–liquid partitioning to an aliphatic alcohol. 1-Octanol provided efficient removal of coextractants, whilst there appeared to be no loss of analytes. In addition, the small amounts of residual 1-octanol dissolved in the aqueous phase of the sample did not disturb the chromatography or the ionization process.

3.2. Liquid chromatographic separation

This study employed a similar LC system to that described previously for the assay of melagatran [15]. Melagatran and OH-melagatran eluted at approximately 1.4 min, and ximelagatran and ethyl-melagatran at approximately 1.9 min. Detection was divided into two time periods, with two compounds and two internal standards in each period. The short analysis time per sample allowed the injection of 15–20 samples/h.

Ion chromatograms for melagatran, OH-melagatran, ethyl-melagatran and ximelagatran extracted from plasma, after an oral dose of ximelagatran, are shown in Fig. 2, together with the four isotope-labelled internal standards. All labelled internal standards contained a minor amount of the respective unlabelled compound. The largest proportion of unlabelled standard was found with melagatran, which resulted in a blank value of about 1 nmol/l, leading to a positive intercept in the calibration curve.

The chromatographic system showed good stability, and performance variations encountered previously with the LC–MS method developed for melagatran [15] did not occur. This may be due to two factors, firstly the injected sample contained formic acid and was thus similar to the mobile phase and secondly the injection volume was lower (10 μl as opposed to 30 μl).

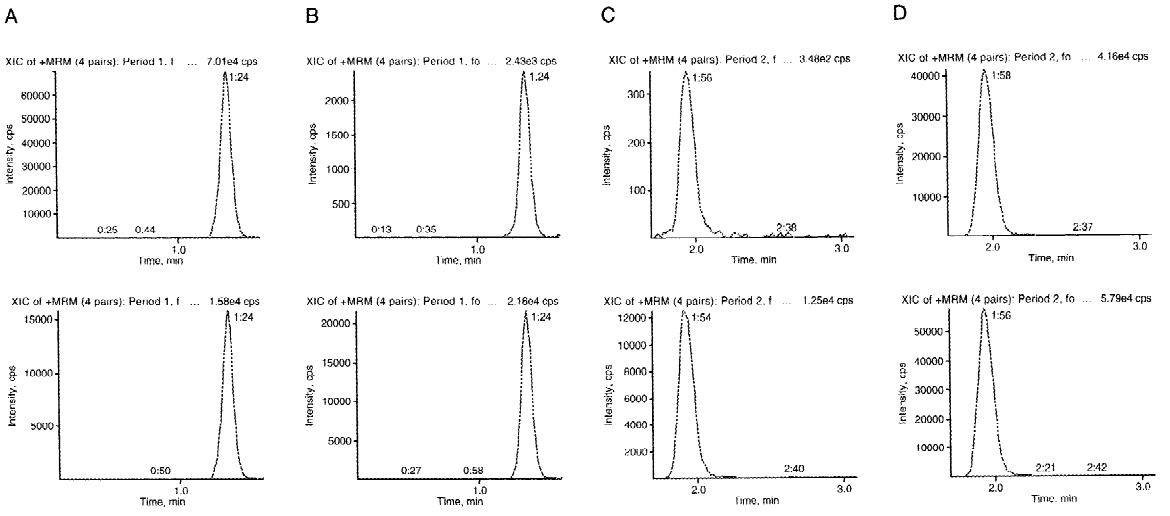


Fig. 2. LC–MS analysis of a human plasma sample taken 2 h after oral administration of ximelagatran, 36 mg. Analytes (upper traces) and individual internal standards (lower traces). Sample concentration (A) melagatran, 1040 nmol/l; (B) OH-melagatran, 34 nmol/l; (C) ethyl-melagatran, <LOQ; and (D) ximelagatran, 216 nmol/l. Conditions: isocratic LC, column C_{18}, 5 μm, 100×4 mm I.D., mobile phase acetonitrile–4 mmol/l ammonium acetate (35:65, v/v) containing 0.1% formic acid, flow-rate 0.75 ml/min, injection volume 10 μl. MS instrument: API 3000. SRM transitions (Table 1). (A) and (B) were recorded in period 1 (time 0–1.7 min), and (C) and (D) were recorded in period 2 (time 1.7–3.1 min). Dwell times were 200 ms for analytes and 100 ms for internal standards.

3.3. MS detection

All analytes were readily detected by positive ion electrospray ionization–MS. The predominant species in all cases was the molecular ion $(M+H)^+$. Product ion spectra of the four analytes and the isotope-labelled internal standards are shown in Figs. 3 and 4, respectively. The SRM transitions used for quantification are listed in Table 1. For ximelagatran and its internal standard, a product ion was chosen which carried the ^{13}C and D isotope labels, resulting in different m/z values for the product ions. The selected product ions used for the other analytes did not carry the isotope labels, resulting in common product ions for analytes and internal standards. Whilst, owing to the short hold-up time of ions in the collision cell, crosstalk between monitored transitions with common product ions does not present a problem when using the API 3000 instrument, measures had to be taken to eliminate crosstalk on the API 365 instrument. While an optional software-controlled collision cell (Q2) purge function was triggered between those transitions involving the same m/z value for the product ion, dummy transitions were introduced where needed, in order to trigger Q2 purge before each of the transitions used for monitoring.

3.4. Linearity and calibration

Standard curves consisting of six plasma concentrations of each of the four analytes, performed in triplicate, demonstrated linear correlation (inaccuracy <15%), in the concentration range used for human plasma samples (10–4000 nmol/l). Repeatability, estimated at three different plasma concentrations ($n=8$), showed a relative standard deviation (RSD) of <10% at 15 nmol/l and <5% at concentrations over 100 nmol/l. The limit of quantification (LOQ) was set at 10 nmol/l (RSD <20%). Reproducibility, or between-day repeatability, was assessed and evaluated by running quality control samples ($n=2$) with each batch of study samples. On 56 occasions, comprising a period of analysis for a clinical study, the mean value obtained with these quality control samples was in the range of 95.7–100.4% of the expected result at 200 nmol/l, with an RSD of 2.6–5.1%. These data for 112 quality control samples in plasma cover all four analytes evaluated on each occasion.

Daily calibration of the analytical method was performed using 10 and 400 nmol/l plasma standard concentrations of each of the four analytes, $n=6$ at each concentration. Full standard curves with $n=3$ at each of six concentrations were run intermittently to ensure linearity.

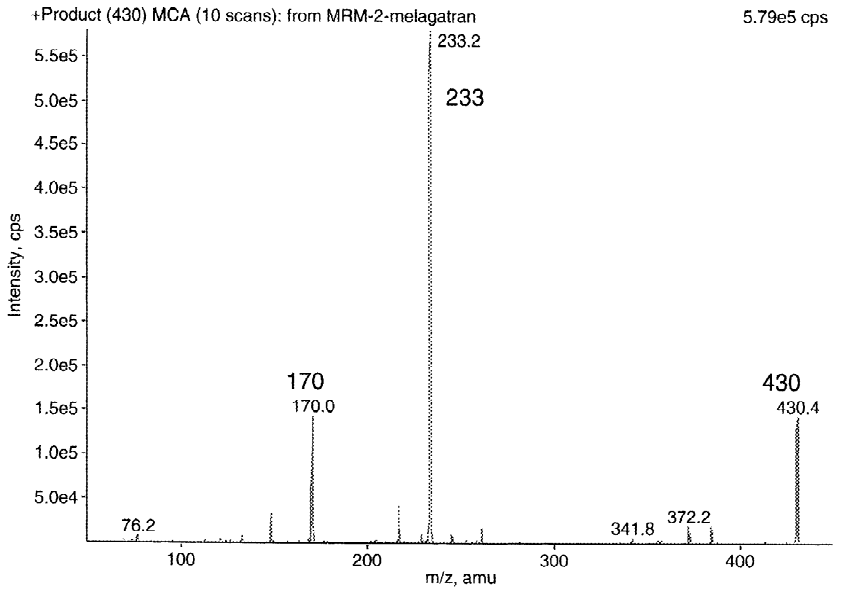
The method presented here was intercalibrated using the earlier published method [15] for the determination of melagatran in 11 authentic plasma samples, without any significant difference in the range 10–400 nmol/l.

3.5. Stability in plasma

When stored at 4–8 °C, standard solutions of the four analytes in the μmol/l range, diluted in HCl (0.01 mol/l), were found to be stable for at least 1 month, individual stock solutions for at least 4 months and melagatran for at least 13 months. Low concentration standard solutions (100–150 nmol/l in 0.01 mol/l HCl) were stable for at least 1 week and were prepared on a weekly basis. The stability at room temperature, of analytes added to human whole blood, was more than 1 h for ximelagatran and 4 h for the other three analytes. The results were the same irrespective of whether heparinised or citrated blood samples were used.

No degradation of any of the analytes was observed after storage of fortified human plasma samples at −20 °C for 12 months. This was also the case for authentic human plasma samples stored at −20 °C for 12 months, as well as for plasma samples subjected to three freeze–thaw cycles. Melagatran and OH-melagatran were stable for at least 20 h, and ethyl-melagatran and ximelagatran for at least 3 h in fortified human plasma samples at room temperature. Quantitative results obtained with processed authentic plasma samples and standards stored at room temperature for 42 h using freshly prepared standard samples for calibration agreed well with those obtained originally. Processed authentic plasma samples stored at 4–8 °C for 8 days also gave quantitative results that agreed with samples processed on day 0.

A



B

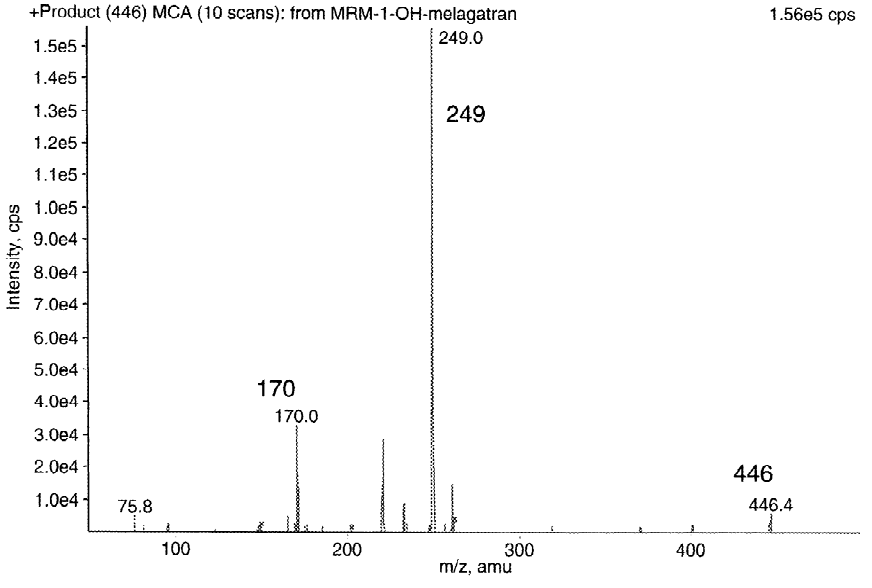
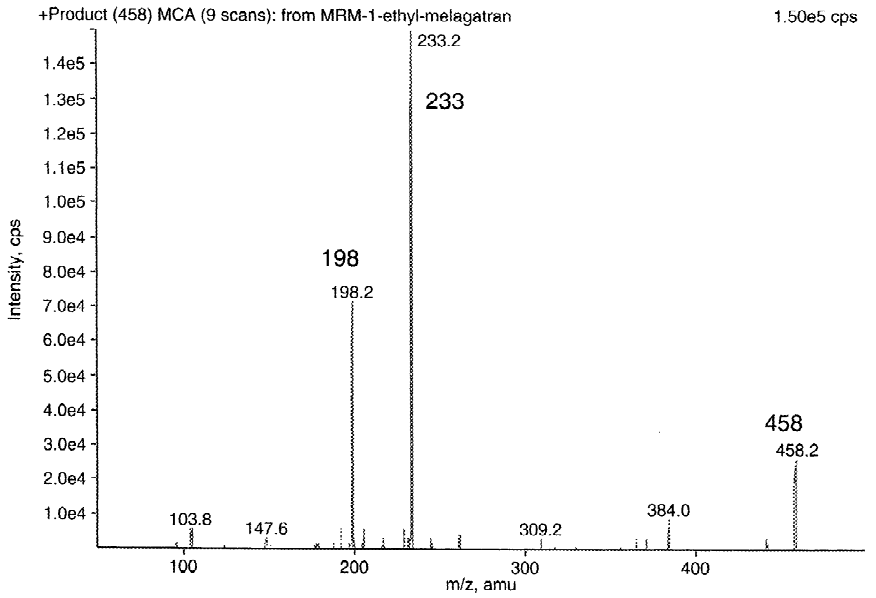


Fig. 3. Product ion spectra of (A) melagatran, (B) OH-melagatran, (C) ethyl-melagatran and (D) ximelagatran. Precursor ion: molecular ion $(M+H)^+$.

C



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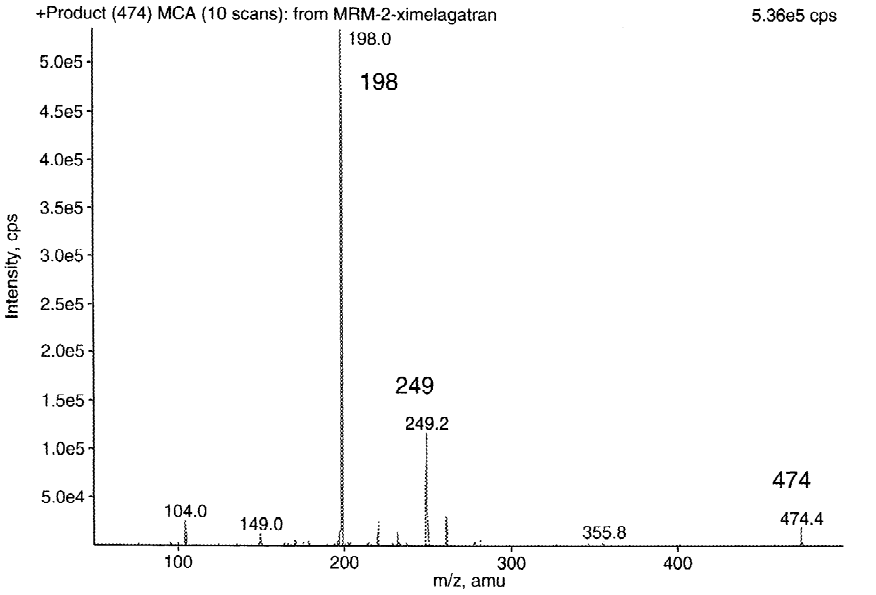


Fig. 3. (*continued*)

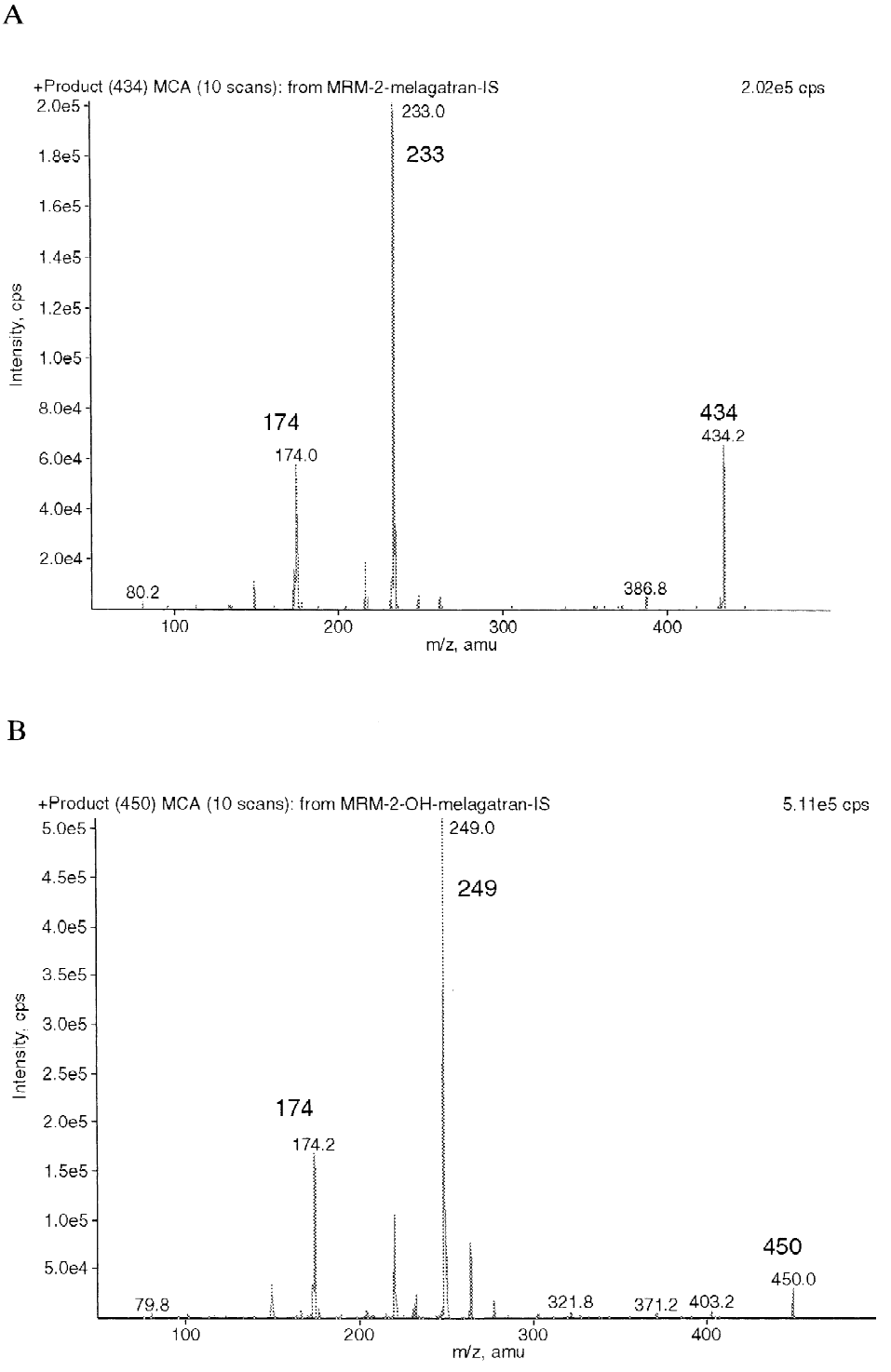
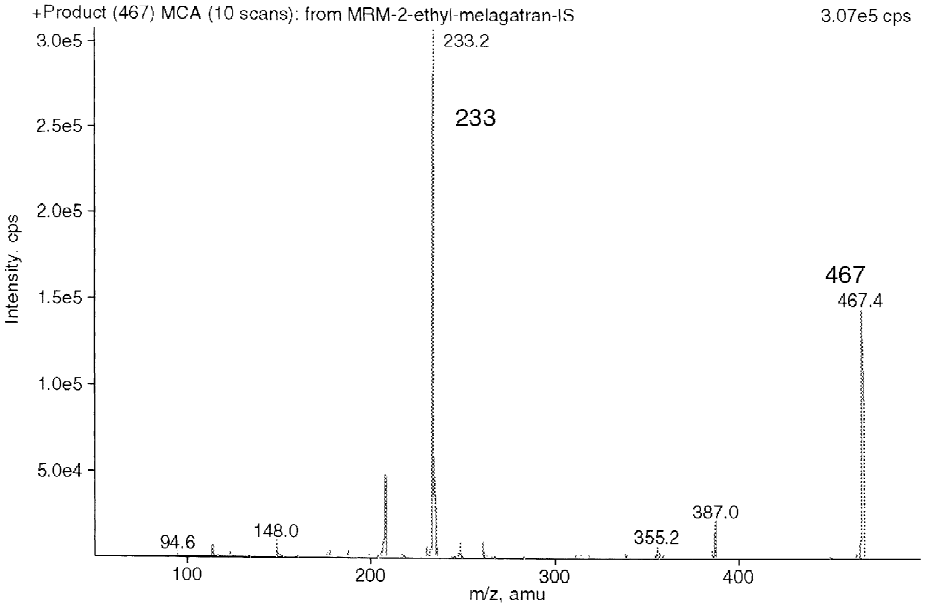


Fig. 4. Product ion spectra of isotope-labelled internal standards (A) melagatran-$D_2^{13}C_2$, (B) OH-melagatran-$D_2^{13}C_2$, (C) ethyl-melagatran-$D_7^{13}C_2$ and (D) ximelagatran-$D_7^{13}C_2$. Precursor ion: molecular ion $(M+H)^+$.

C



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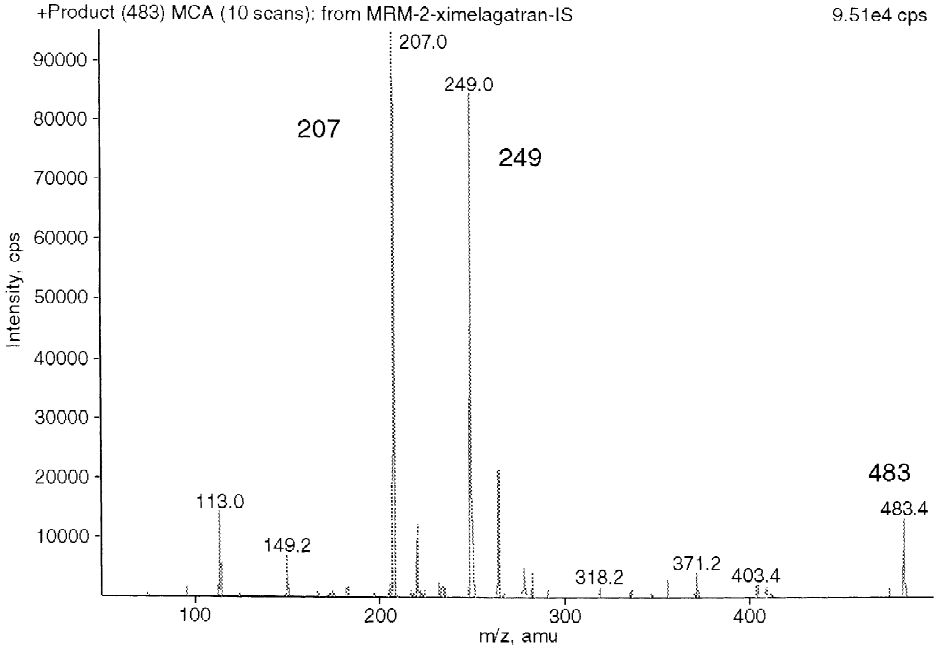

Fig. 4. (*continued*)

3.6. Stability in urine

All four compounds were stable in authentic urine samples for at least 5 months when stored at −20 °C, and for at least 6 h at room temperature. No degradation was found after three freeze–thaw cycles, or in processed samples stored for up to 8 days at 4–8 °C.

3.7. Analysis of urine, breast milk and whole blood

Analytical methods were developed for determining the four analytes in other biological matrices: human urine and breast milk plus whole blood from rat or mouse. Isotope-labelled internal standards were used in these methods as well. Ion chromatograms from these four sample matrices did not deviate in a qualitative manner from those of human plasma samples (Fig. 2), data are therefore not shown.

Human urine samples were analysed with gradient elution LC–MS after simple dilution with 10% acetonitrile in acetate–formic acid buffer solution. The gradient elution was aimed at removing not only early eluting but also late eluting high concentration components which otherwise could generate ion suppression in subsequent sample runs. Repeatability was estimated for the four analytes at three different concentrations in urine ($n=10$). The RSD was 5% or lower at concentrations ≥100 nmol/l. RSD was 4–5% for quality control samples ($n=28$) at 2000 nmol/l. The lower LOQ was set at 100 nmol/l (100 μl sample volume).

After removal of excess fat, human breast milk samples were analysed using the same SPE procedure as described for plasma, but with the omission of the 1-octanol extraction. It was found, from test samples fortified with analytes, that removal of the excess fat did not decrease analyte recovery or the concentrations measured. Only the API 3000 instrument was used for this application, giving an estimated lower LOQ of 2 nmol/l (1000 μl sample volume). Repeatability was estimated ($n=10$) with an RSD of 5% or lower at concentrations of ≥10 nmol/l.

The high enzymatic activity of rat and mouse blood resulted in rapid degradation, especially of ximelagatran and ethyl-melagatran thus precluding the use of untreated rat or mouse plasma samples for analysis. This problem was overcome by rapid denaturation of enzymes by mixing whole blood with SDS immediately at collection. Simultaneously, SDS also caused total haemolysis. Samples were further stabilised by storage at −70 °C. Attempts to use Bond Elut C_8 and other silica-based reversed-phase SPE materials for extraction of analytes resulted in column clogging and low recoveries. However, the polymeric SPE material OASIS could be used without clogging, and satisfactory recoveries were obtained for ximelagatran, ethyl-melagatran and melagatran. OH-melagatran was not retained quantitatively without compromising the extensive washing necessary for removal of SDS and other matrix components prior to elution. The same type of LC column as used for human plasma was employed, with slightly modified elution conditions using an acetonitrile step gradient. The achieved precision was lower than in the assay for human plasma samples, but was quite adequate for exploratory studies in rat and mouse. The lower LOQ was estimated at 15 nmol/l for ximelagatran, 25 nmol/l for ethyl-melagatran and 40 nmol/l for melagatran (200 μl sample volume).

4. Conclusions

In the present study we have developed analytical methods for the quantification of ximelagatran, melagatran and the intermediate metabolites, OH-melagatran and ethyl-melagatran, in human plasma and other biological matrices. After sample clean-up, LC–MS allowed rapid analysis with high accuracy, reproducibility and precision for the four analytes, promoted by the use of an isotope-labelled internal standard for each analyte. The analytical method for plasma has now been used to determine both ximelagatran and melagatran in thousands of samples in human pharmacokinetic and clinical studies, while the methods described for the other biological matrices have been successfully applied in other separate studies.

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