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Determination of ximelagatran, melagatran and two intermediary metabolites in plasma by mixed-mode solid phase extraction and LC–MS/MS

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Abstract

An analytical method was developed for the determination of ximelagatran, an oral direct thrombin inhibitor, its active metabolite melagatran, and the two intermediate metabolites, OH-melagatran and ethyl-melagatran in human plasma. Extraction of plasma was carried out on a mixed mode bonded sorbent material (C8/SO_3^-). All four analytes, including their isotope-labelled internal standards, were eluted at high ionic strength with a mixture of 50% methanol and 50% buffer (0.25 M ammonium acetate and 0.05 M formic acid, pH 5.3) with an extraction recovery above 80%. The extracts were demonstrated to be clean in terms of a low concentration of albumin and lysoPC. The sample extraction was fully automated and performed in 96-well plates using a Tecan Genesis pipetting robot. Analysis of the extracts were performed with liquid chromatography followed by positive electrospray ionization mass spectrometry. The low organic content and the low pH of the extracts allowed for, after dilution 1:3 with buffer, direct injection onto the LC-column. The four analytes were separated on a C18 analytical LC-column using gradient elution with the acetonitrile concentration varying from 10 to 30% (v/v) and the ammonium acetate and acetic acid concentration kept constant at 10 and 5 mmol/L, respectively, at a flow rate of 0.75 mL/min. Linearity was achieved over the calibrated range 0.010–4.0 μmol/L with accuracy and relative standard deviation in the range 96.9–101.2% and 6.6–17.1%, respectively at LLOQ, and in the range 94.7–102.6% and 2.7–6.8%, respectively at concentrations above 3 × LLOQ. The method replaces a manual method, and displays the advantages of having a fully automated sample clean-up, no evaporation/reconstitution step, high recovery, and complete LC-separation of all four analytes.

Keywords: Thrombin inhibitor; Mixed-mode solid phase extraction

1. Introduction

Ximelagatran (Exanta, AstraZeneca, Mölndal, Sweden) is a direct oral thrombin inhibitor, which was extensively investigated for use as an anticoagulant and antithrombotic agent in prophylaxis and treatment of various thromboembolic conditions [1–10]. Ximelagatran is rapidly absorbed and converted to melagatran, the active form and major metabolite in plasma [11,12].

An earlier developed method for determination of ximelagatran, melagatran, and two intermediary metabolites [13] is based on solid-phase extraction (SPE) on octylsilica, including a solvent evaporation step and a final liquid–liquid extraction step, where *n*-octanol is used to remove late eluting matrix components, (lysoPC), that could cause ionization suppression. For one of the intermediary metabolites (ethyl-melagatran), recovery is only around 60%, while recoveries for the others are around 80% or higher. The four analytes are not completely separated on the LC system.

This paper describes a new improved method aimed for high extraction recoveries for all analytes, a simple sample treatment protocol allowing full automation, and complete LC separation of all four analytes. Considering that the analytes carry charged as well as hydrophobic functional groups, mixed-mode solid

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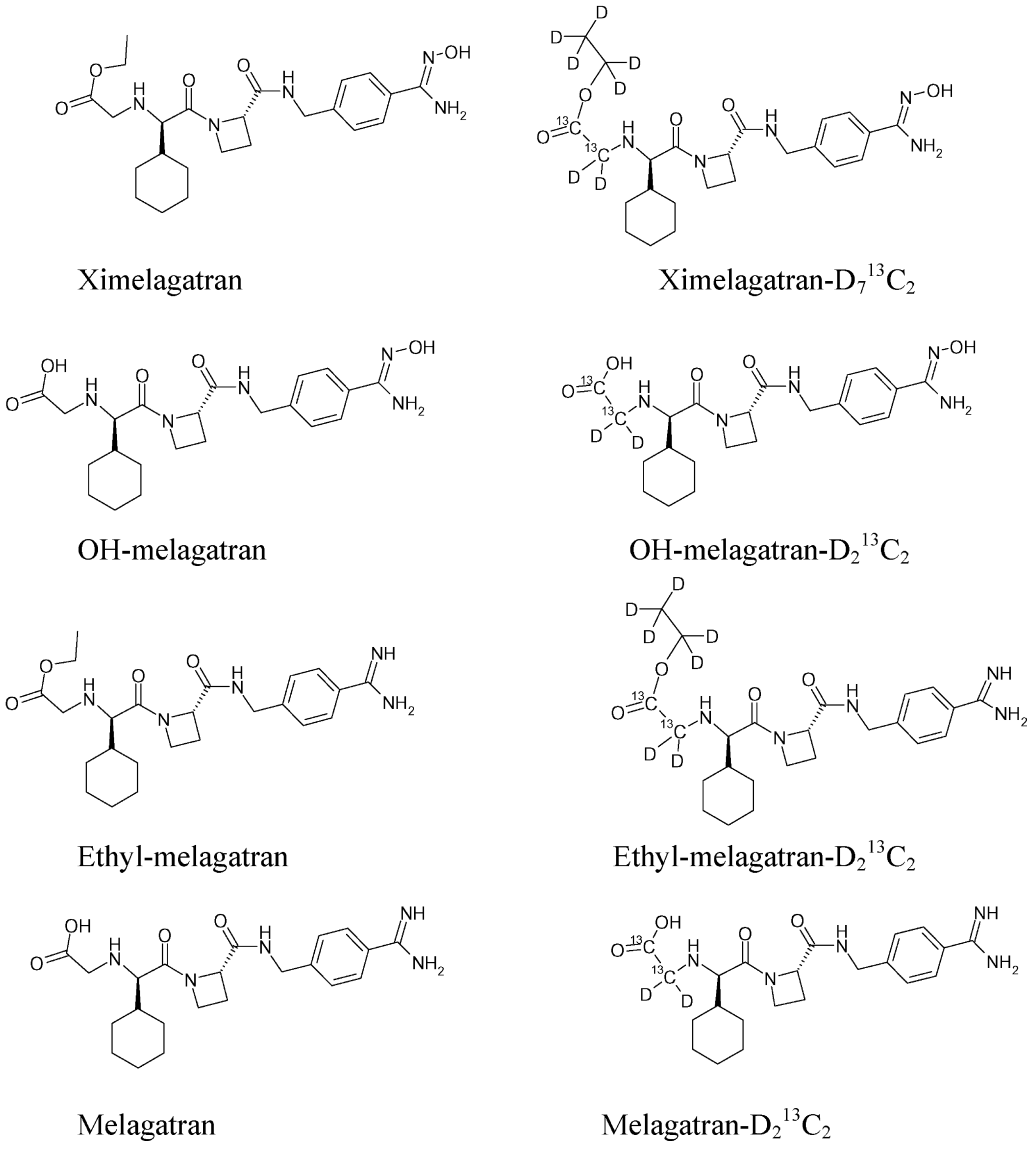

Fig. 1. Structures of ximelagatran, intermediate metabolites OH-melagatran and ethyl-melagatran, and melagatran and their corresponding isotope-labelled internal standards.

phase extraction was judged as an appropriate technique for sample preparation [14]. Compared with traditional reversed-phase SPE, mixed-mode SPE with an additional washing step may render cleaner extracts. However, retention of analytes must allow the simultaneous recovery of all four analytes, which have in common a secondary amino group but differ in terms of other protolytic groups (see Fig. 1). If the analytes are chromatographically resolved, a possible risk of disturbances due to conversion of analytes during detection is eliminated. Also interference from co-eluting analytes on ionisation efficiency is avoided.

After the new method was developed and used in many clinical studies including several thousands of samples, ximelagatran was withdrawn from further clinical development in February 2006 due to concerns over liver safety. However, ximelagatran may still be regarded as an interesting reference substance in the tromboembolic research field.

2. Experimental

2.1. Chemicals and materials

Ximelagatran (pK_a values 5.2 and 4.5), OH-melagatran, ethyl-melagatran, melagatran (pK_a values 2.0, 7.0 and 11.5) and their respective deuterium (D) and ^{13}C-labelled internal standards (Fig. 1) were supplied by AstraZeneca R&D, Mölndal, Sweden. Methanol and acetonitrile of HPLC grade were obtained from Rathburn (Walkerburn, UK). Acetic acid, ammonium hydroxide solution, formic acid, and hydrochloric acid (Titrisol) of analytical grade were obtained from Merck (Darmstadt, Germany) and ammonium acetate, ammonium formate, and ammonium hydrogen carbonate of pro analysis grade from Sigma–Aldrich (Steinheim, Germany). High purity water was obtained from an Elga purification system (High Wycombe, UK). Solid-phase extraction tubes were Isolute Array HCX,

Isolute Array HXC3, and Isolute Array HCX5, 1 mL, 25 mg purchased from IST Isolute (International Sorbent Technology, Hengoed, UK).

2.2. Blood collection

Blood samples were collected into tubes containing 10% of 0.12 mol/L trisodium citrate solution as anticoagulant. Plasma was separated by spinning the blood for 10 min at 1500 × *g* within 1 h of sampling. The plasma was then transferred to polypropylene cryo-vials and stored frozen at −20 °C until analysis.The dilution of the plasma due to the citrate solution is estimated at 1.185 and measured concentrations have to be corrected for this dilution.

2.3. Pipetting robot

The SPE system consisted of a robotic sample processor, Genesis RSP 150 (Tecan, Hombrechticon, Switzerland), a manifold (Tomtec Inc., Hamden, MA, USA) and an on-site assembled ejector pump for underpressure control.

2.4. Preparation of standard solutions and spiked plasma standards

Standard solutions of the analytes were prepared in 0.01 mol/L HCl to a concentration of 34 μmol/L and 0.084 μmol/L for each analyte. Stock solutions of the internal standards were prepared in 0.01 mol/L HCl, and then further diluted with 0.25 mol/L HCl to give concentrations in the range of 0.5–0.8 μmol/L (concentrations in this range were found to give suitable responses) for all four internal standards.

The robotic sample processor prepared plasma standards by pipetting 100 μL drug-free citrated plasma to separate glass tubes, followed by 65 μL standard solution, and an additional aliquot of 485 μL drug-free citrated plasma. The content of the tubes was mixed by repeated aspirating and dispensing back.

Prior to mixing, an extra volume of the spiked plasma was aspirated in order to serve as a shielding volume between the system liquid and the sample. In this way, dilution of the spiked plasma sample with system liquid was avoided. After mixing, this extra volume was dispensed into the waste.

2.5. Analytical procedure

The plasma samples were thawed at room temperature, homogenised by vortex-mixing and spun in a centrifuge for 5 min at 2800 × *g*. The robotic sample processor distributed the liquids in the SPE steps. The extraction was performed on Isolute Array 1 mL HCX, 25 mg SPE-columns. The SPE columns were activated with 500 μL methanol, the pressure level in the manifold was adjusted to 7 mbar below ambient pressure and the columns were conditioned with 250 μL water. In the sample load step, 250 μL of 0.25 mol/L HCl (used to adjust the pH of the plasma sample to a pH of 1), containing the internal standards, and 250 μL plasma were loaded simultaneously on the SPE-columns. Washing with 500 μL acetic acid, 1 mol/L, was followed by washing using 500 μL methanol. After this second washing, the underpressure was released to ambient, the waste plate removed and a collection plate inserted in the manifold. The pressure was again adjusted to 7 mbar below ambient pressure and the robotic sample processor delivered the eluent. The analytes were eluted with two portions (2 × 250 μL) of a mixture of 50% methanol and 50% buffer (0.25 M ammonium acetate and 0.05 M formic acid, pH 5.3). After the elution step was completed, the collection plate was sealed, mixed and spun in a centrifuge for 5 min at 2800 × *g*. An aliquot of 150 μL was transferred to another plate and diluted with 300 μL of the diluent; 0.01 M ammonium acetate in 0.008 M formic acid, pH 6.9, resulting in final concentrations of metanol and buffer of 16.7 and 83.3% (0.058 M ammonium acetate and 0.014 M formic acid), respectively. This plate was also sealed, mixed and spun as described above. The plate was placed in the autosampler and 30 μL of the diluted extract was injected onto the chromatographic system.

2.6. Liquid-chromatography–mass spectrometry

The liquid chromatographic system consisted of a Perkin-Elmer 200 series autosampler and LC pumps (Überlingen, Germany). The mass spectrometer was a API 3000 triple quadrupole with electrospray (turbo-ion spray) interface (Sciex,Concord, Canada). The effluent from the LC column (flow-rate 0.75 mL/min) was split with a Valco T-connection (Valco International, Schenkon, Switzerland). Data were processed and evaluated on Analyst version 1.2 software from Applied Biosystems MDS Sciex (Foster City, USA). LC separations were undertaken at 40 °C on a reversed-phase Hypersil HyPURITY C_{18} 5 μm 180 Å analytical column (100 mm × 2.1 mm i.d.) from Thermo (Runcorn, UK).

Mobile phase A consisted of 10% acetonitrile and 90% buffer (10 mmol/L ammonium acetate and 5 mmol/L formic acid, pH 5) and mobile phase B consisted of 50% acetonitrile and 50% buffer (10 mmol/L ammonium acetate and 5 mmol/L formic acid, pH 5).The elution of melagatran and OH-melagatran was made mainly under isocratic conditions (10% acetonitrile, mobile phase (A) which was followed by a step-gradient to 30% acetonitrile (50% mobile phase A and 50% mobile phase (B) after 1.4 min. This gave retention times of approximately 1.3 min for melagatran, 2.1 min for OH-melagatran, 2.7 min for ethyl-melagatran and 3.2 min for ximelagatran with a flow-rate of 0.75 ml/min. The isotope-labelled internal standards eluted with a retention time difference to their respective analytes of 0.03 min or less.

The liquid flow to the mass spectrometer was 150 μL/min. Typical settings for declustering potential were 35–46 V, turbo heater temperature was 375 °C and collision energy was 25–33 eV (Table 1). 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 measured in unit mass resolution mode. The SRM transitions of the precursor ions $(M+H)^+$ and the corresponding product ions are shown in Table 1. Product ions were monitored in three

Table 1
Selected reaction monitoring (SRM) transitions used for quantification and mass spectrometric settings

Compound	Precursor ion (m/z) $(M+H)^+$	Product ion (m/z)	Declustering potential (V)	Collision energy (eV)	Dwell times (ms)
Melagatran	430	233	66	33	300
Melagatran–$D_2{}^{13}C_2$	434	233	66	33	150
OH-melagatran	446	249	61	29	150
OH-melagatran–$D_2{}^{13}C_2$	450	249	61	29	75
Ethyl-melagatran	458	233	46	25	150
Ethyl-melagatran–$D_7{}^{13}C_2$	467	233	46	25	75
Ximelagatran	474	198	36	33	150
Ximelagatran–$D_7{}^{13}C_2$	483	207	36	33	75

time periods to allow for longer dwell times. A dwell time of 150–200 ms was used for the analytes and 75–100 ms was used for the internal standards (Table 1).

2.7. Measurement of albumin and lysoPC

Collected fractions from SPE experiments were separated on a Zorbax 300 Extend C18 column (3 mm × 30 mm, 3.5 μm, 80 Å, Agilent Technologies) at a flow rate of 0.6 mL/min by a 6–76% acetonitrile gradient in 20 mM ammonium hydroxide (pH 10.8). Serum albumin was detected at 280 nm. Serum albumin (7 μmol/L) in 20 mM ammonium acetate with 20% acetonitrile was used as working standard solution. Selected-ion monitoring of lysophosphatidylcholine (lyso PC) 16:0 at m/z 496 was accomplished by adding formic acid post-column to the flow entering the electrospray ion source. Estimation of relative amounts of phospholipid in samples was based on measured peak area.

2.8. Daily calibration and method performance

Daily calibration of the analytical method was performed using 0.010 and 4.0 μM plasma standard concentration of each of the four analytes, $n=6$ at each concentration. The relative peak areas of the plasma standards for the analytes over the internal standards were used for calculation of the concentration in the study samples by linear regression with no weighting. Full standard curves with $n=4$ at each of six or seven concentrations were run in predetermined intervals to ensure linearity.

3. Results and discussion

3.1. Extraction

A set of experiments were conducted to find the optimal conditions for achieving high recoveries combinded with clean extracts when isolating all four analytes from plasma on mixed mode columns. All experiments were performed on three types of mixed mode SPE-columns differing in the length of the hydrocarbon chain (C4 (Isolute HCX5), C8 (Isolute HCX) or C18 (Isolute HCX3)). The results showed only minor differences between these SPE-phases and Isolute HCX was chosen for the analytical procedure.

To evaluate the effect of ion-exchange interactions during sample application, the recovery was studied as a function of the plasma pH. Plasma samples were examined at pH 1.1, 2.7, 4.0, 7.7 and 9.5. High recoveries (>80%) were generally obtained with moderately higher values at the low and high ends of the tested pH interval. One lower recovery (59%) was seen for OH-melagatran on Isolute HCX5 at pH 9.5.

The percentage of analyte that was lost during the methanol wash was also measured. The loss was most pronounced at pH 4, and always on the SPE-column with the longest alkyl ligand, and was less than 7% for melagatran and OH-melagatran, and less than 15% for the more hydrophobic analytes ximelagatran and ethyl-melagatran. No effect was seen from reducing the methanol concentration to 30%.

In summary, the pH of the plasma did not exert any major influence on the recovery and the small effect that was noticed did not seem to be related to the number of charged groups of the analytes (Table 2). The somewhat reduced recoveries in the mid pH range seem to be caused by loss of analyte when washing the SPE-column with methanol, rather than failure to trap the analyte during the sample application. However, charge repulsion is presumably the explanation to the lower recovery of the negatively charged OH-melagatran at pH 9.5 on Isolute HCX5, the SPE-column with the shortest alkyl ligand. Consequently, in this case a very low amount of analyte was seen in the wash extract.

The results of the experiment indicate that the hydrophobic mechanism dominates when trapping the analytes, and it may be that the ionic strength of the plasma sample is too high to allow retention by ion-exchange. For the final method, a pH in the sample application step of about 1 was chosen, a pH at which all four analytes are positively charged (Table 2), and the carboxylic acid group of melagatran and OH-melagatran is protonized.

Table 2
Number of positively (+) and negatively (−) charged groups at different pH:s

pH	Melagatran	OH-melagatran	Ethyl-melagatran	Ximelagatran
1.1	++	+	++	++
2.7	−++	−+	++	++
4.0	−++	−+	++	++
7.7	−+	−	+	No charged groups
9.5	−+	−	+	No charged groups

During the methanol wash step, the analytes are held primarily by ion-exchange interaction as cat-ions, and the wash can thus be done with pure methanol solvent without eluting the analyte. As a measure of the efficiency of the methanol wash step, the concentration of albumin, the major protein in plasma, and the concentration of lysoPC, a lipophilic plasma component known to give rise to ion suppression in the mass spectrometer [15,16], which was observed in the previous method [13], were determined in both the wash extract and the final eluate. Washing with pure methanol as well as 30% methanol was performed.

A small amount of albumin, approximately 2 μM, corresponding to approximately 0.3% of the plasma concentration, was found in the wash extract, while in the final eluates the concentration was below the detection limit (2 μM) regardless of the elution conditions used. This indicates that the plasma proteins are either never retained or are permanently bound to the SPE-column.

The concentration of lysoPC was more than 40 times higher in the wash extract than in the final eluate when washing was performed with 100% methanol. It can therefore be concluded that the amount of lysoPC in the final eluate corresponds to less than 2% of the amount in the original plasma sample. Reducing the methanol concentration to 30% greatly reduced the amount of lysoPC eluted during the washing step, leaving the major part of the lysoPC remaining on the column. However, the concentration in the final eluate was comparable with the concentration found when washing with pure methanol and thus, the lysoPC did not elute under the condition used.

Elution from a mixed mode sorbent must be done with a solvent mixture that minimizes both hydrophobic and ion-exchange interactions. Elution was first done by applying the common procedure of eluting at high pH with methanol containing 5% aqueous ammonium hydroxide. This resulted in a low recovery (28.7%) of ethyl-melagatran, while the recoveries of the other three analytes were more than 80%.

Eluting the analytes with a methanol-based elution liquid with high ion strength at low pH was investigated. The result of varying the concentration of ammonium acetate in the buffer

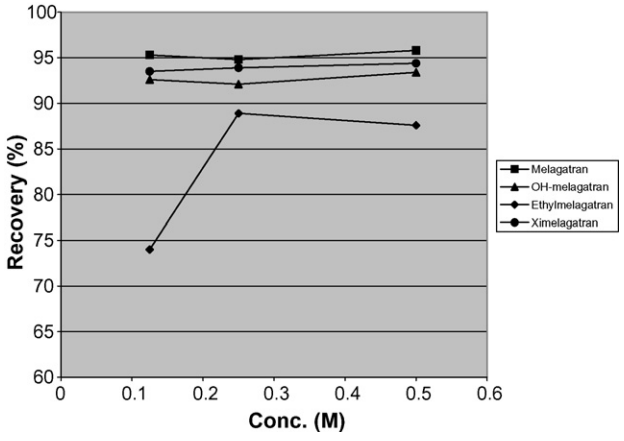

Fig. 2. Recovery on Isolute HCX plotted as a function of the ammonium acetate concentration in the elution buffer. The elution liquid was a mixture of 50% methanol and 50% buffer (0.125–0.5 M ammonium acetate and 0.05 M formic acid, pH 4.7–5.6).

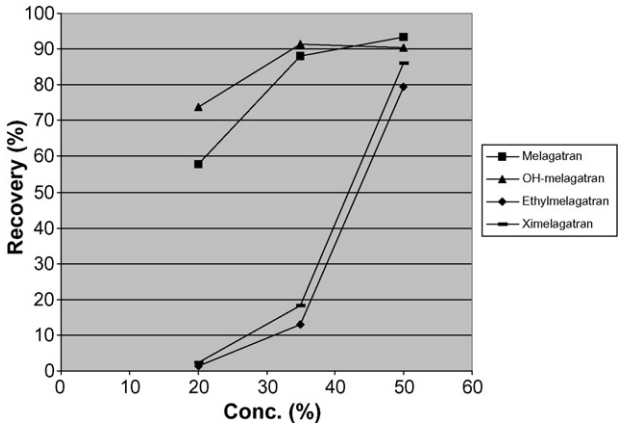

Fig. 3. Recovery on Isolute HCX plotted as a function of the methanol concentration in the elution liquid. The final concentration of ammonium acetate and formic acid in the elution liquid was 0.125 and 0.025 M, respectively, pH 5.3, $n = 2$.

component of the elution liquid is illustrated for Isolute HCX in Fig. 2. The effect on the recovery from varying the methanol concentration is shown for Isolute HCX in Fig. 3. Ethyl-melagatran displayed the strongest retention, but could be eluted with a mixture of 50% methanol and 50% buffer (0.25 M ammonium acetate and 0.05 M formic acid, pH 5.3) and this composition of the elution liquid was chosen for the final method. After addition of two parts of an aqueous diluent solution and mixing, the extracts could be injected onto the chromatographic system.

The extraction recoveries, measured at the concentration levels 0.01, 0.2 and 4 μmol/L ($n = 3$) by comparing the response from spiked plasma samples with the response for blank plasma samples spiked to the corresponding concentrations after extraction, were in the range 91–95% for melagatran, 89–92% for OH-melagatran, 81–91% for ethyl-melagatran and approximately 85% for ximelagatran.

From the results described above, it appears that sample clean-up on mixed mode SPE-columns may be an option for compounds which, when eluted from conventional reversed phase SPE-columns, require such a high organic concentration that they coelute with the most lipophilic plasma components. Furthermore, for basic compounds that are charged even at high pH, elution at high ion strength from mixed mode SPE-columns may be preferable to the more common procedure of eluting with methanol containing ammonium hydroxide. The former approach also allows elution with low percentage organic solvent and this facilitate injection into LC without prior evaporation.

3.2. Chromatographic separation

Melagatran and OH-melagatran were not fully separated in the previous method [13], and accordingly different stationary phases and mobile phase compositions were tested in the present investigation. It was found that by increasing pH from 2.9 to 5.0 using an ammonium acetate–acetic acid buffer and 10% acetonitrile as organic modifier (Fig. 4), the separation factor was improved from 1.1 to 1.9 on a stationary phase of Hypersil HyPURITY C18, which was chosen

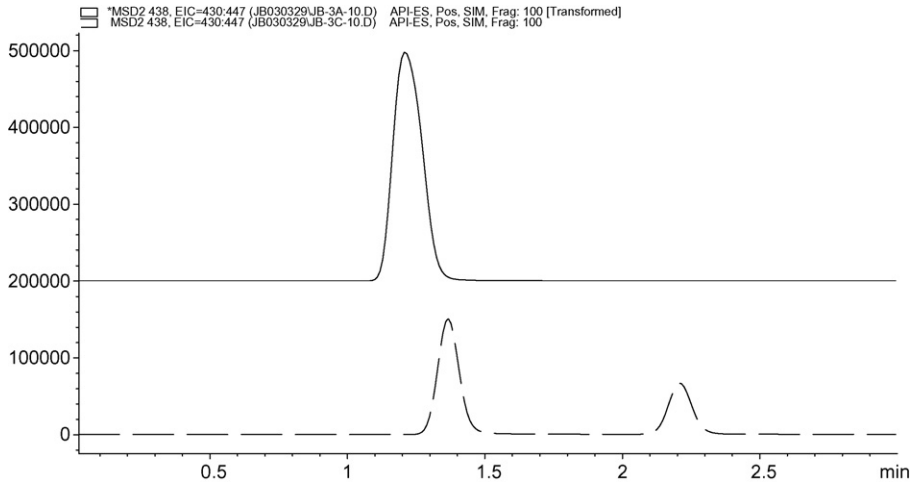

Fig. 4. Separation of melagatran and OH-melagatran using two different mobile phases, 10% acetonitrile in 10 mmol/L formic acid, pH 2.9 (upper) and 10% acetonitrile in 10 mmol/L ammonium acetate and 5 mmol/L acetic acid, pH 5.0 (lower) with HyPurity column.

out of a number of columns of comparable properties, judged to have small batch varation, high retention and minimized tailing for basic compounds. Peak symmetry was quite satisfactory for most separation systems tested. Temperature was raised to 40 °C to promote column efficiency and to lower column back-pressure. A pressure of approximately 2900 psi was observed. No effect on lifetime from elevated temperature or pressure was noted, which is consistent with litterature data [17,18]. The more hydrophobic compounds, ethylmelagatran and ximelagatran did not elute within reasonable time using isocratic elution with 10% acetonitrile why a step-gradient to 30% acetonitrile was introduced after 1.4 min when the first two components were separated. All four compounds were then eluted within 3.5 min from the chromatographic column as shown in Fig. 5.

3.3. Matrix effects on ion suppression

A solution of 0.5 μmol/L of each of the four analytes was continuously infused via a T-connection into the effluent from the LC-column at a flow rate of 50 μL/min. Blank plasma extract or pure solvent was injected and eluted according to the final metod. The ion intensities were monitored in selected reaction mode, and a difference in signal intensity was interpreted as ion suppression. No differences were seen for any of the four analytes when comparing signal traces from injection of blank matrix and pure solvent.

3.4. Performance of method

Six replicates at each of seven different plasma concentration levels were injected to demonstrate a linear standard curve in the concentration range of 0.010–4.0 μM (Table 3). The lower limit of quantification (LLOQ) was set to 0.010 μM and the upper limit of quantification (ULOQ) was set to 4.0 μM for the API 3000 instrument. With the concentration levels used for ULOQ and LLOQ, the calibration range completely covered the expected therapeutic concentration range of all four analytes. Based on results from clinical studies, the ULOQ was chosen somewhat higher than the highest expected therapeutic concentration. The LLOQ was chosen at a concentration level expected to represent the lowest therapeutic level of interest and at which the accuracy was within 85–115% and the coefficient of variation of the repeatability less than 20%. A chromatogram with added concentrations at LLOQ for the four analytes is shown in Fig. 6.

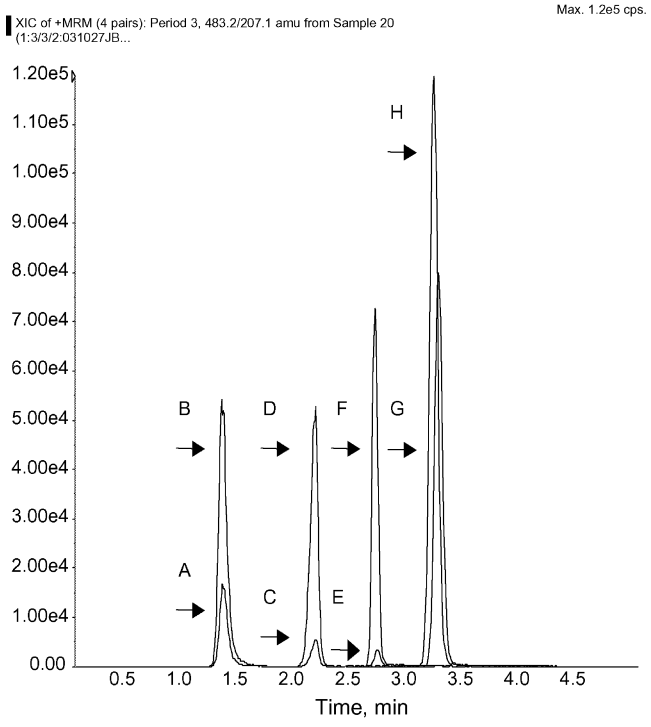

Fig. 5. Chromatograms from an authenic human citrated plasma sample with added internal standard. The sample is collected 30 min after an oral dose of ximelagatran. Concentration of melagatran (A) and its internal standard (B): 0.167 μmol/L and 0.805 μmol/L, OH-melagatran (C) and its internal standard (D): 0.080 μmol/L and 0.749 μmol/L, ethyl-melagatran (E) and its internal standard (F): 0.017 μmol/L and 0.604 μmol/L, ximelagatran (G) and its internal standard (H):0.466 μmol/L and 0.641 μmol/L.

Table 3
Linearity of standard curves in the range 0.01–4 μmol/L shown as accuracy of spiked plasma samples at five different concentration levels

Concentration (μmol/L)	Melagatran		OH-melagatran		Ethyl-melagatran		Ximelagatran	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.01	–[a]	1.8	–[a]	6.1	–[a]	4.7	–[a]	3.4
0.03	93.6	2.9	97.3	2.3	95.7	2.6	99.1	4.3
0.1	97.8	2.7	99.0	2.1	97.8	1.4	100.9	3.5
0.3	96.7	3.8	96.8	3.8	96.6	4.3	99.7	3.7
0.8	98.0	2.4	96.7	3.3	97.8	3.3	100.3	3.1
2	98.9	2.1	97.1	1.4	98.2	1.4	99.9	1.1
4	–[a]	4.0	–[a]	2.9	–[a]	3.9	–[a]	3.7

Six replicates were run at each concentration level, except at 0.01 μmol/L where the number of replicates was five. The levels 0.01 and 4 μmol/l represent the standard concentrations.

[a] As the concentration levels 0.01 and 4 mol/L represent the standards, accuracy cannot be calculated for these levels.

Table 4
Accuracy and precision data (calculated as one-way ANOVA) for the determination of melagatran, OH-melagatran, ethyl-melagatran and ximelagatran from three analytical runs

Compound	Concentration (μmol/L)	Accuracy (%)	Repeatability ($n=6$, $p=3$) (%)	Intermediate precision ($n=6$, $p=3$) (%)
Melagatran	0.00996	100.3	6.6	6.6
	0.0299	98.5	4.6	4.6
	3.980	100.7	3.6	3.6
OH-melagatran	0.00961	100.1	10.1	10.1
	0.0288	99.8	4.8	5.2
	3.840	100.5	4.3	4.3
Ethyl-melagatran	0.00928	96.9	16.9	17.1
	0.0278	94.7	5.8	5.8
	3.710	100.6	3.7	3.7
Ximelagatran	0.00934	101.2	10.7	10.7
	0.0280	100.1	4.2	6.8
	3.730	102.6	2.7	4.8

Reproducibility (intermediate precision) and repeatability were evaluated from three analytical runs ($n=6$, $p=3$) performed on different days (Table 4). Repeatability, estimated at three different plasma concentrations showed a coefficient of variation (CV) of <20% at LLOQ and <10% at ULOQ for all analytes. As the calculated reproducibility was the same or only marginally larger than the repeatability, it can be concluded that the interday variation was small compared with the intraday variation.

Selectivity of the method was confirmed as no interfering peaks were observed when extracting and analysing citrated drug-free plasma samples from six different sources.

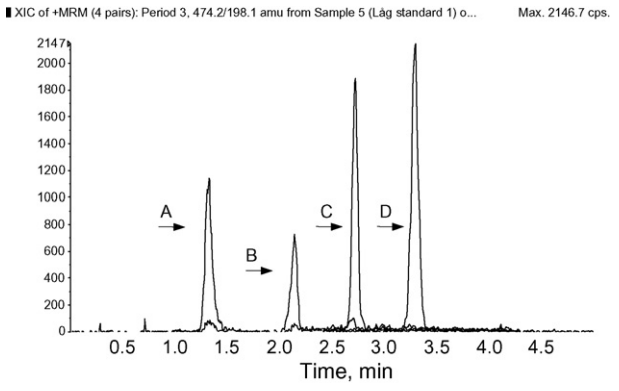

Fig. 6. Chromatogram from a human citrated plasma sample spiked to concentration at lower limit of quantification. Chromatogram from a blank drug-free citrated sample is shown in the same chromatogram and can be seen as background noise. Measured compounds are melagatran (A), OH-melagatran (B), ethyl-melagatran (C) and ximelagatran (D).

3.5. Stability

The stability of spiked ($n=4$) and authentic ($n=20$) processed samples was investigated for 72 h in room temperature and for 2 weeks at 4–8 °C by comparing the results after storage with the results determined on day 0. For spiked samples, two concentration levels representing 3× LLOQ and 0.8× ULOQ were included. All analytes were stable during this time as the concentrations after storage of the spiked processed samples were all within 90–110% of the initial values, and more than 90% of the concentrations of the authentic processed samples were within 80–120% of the initial values.

4. Conclusions

A new method for the determination of ximelagatran, melagatran and two intermediate metabolites was developed. This method represents an improvement of an earlier described

method [13] which is based on non-automated SPE on octylsilica, followed by solvent evaporation and liquid–liquid extraction prior to instrumental analysis. Sample clean-up on mixed mode SPE-columns (Isolute HCX) made it possible to remove highly lipophilic matrix components, (lysoPC), thereby making the liquid–liquid extraction step unnecessary. The use of high ion strength allowed elution at a low concentration of methanol and low pH with high recovery also for the strongly retained ethyl-melagatran. The composition of the elution liquid made it possible to, after dilution, inject the extracts onto the LC-column without a preceding evaporation/reconstitution step. This simplified sample treatment facilitated the adaption of the procedure to an automatic pipetting robot.

By lowering the acetonitrile concentration in the first part of the LC-run substantially as well as increasing the pH in comparison to to the previous method, LC-separation of all four analytes was achieved.

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