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Development and validation of a liquid chromatography–tandem mass spectrometric assay for Eplerenone and its hydrolyzed metabolite in human plasma

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Abstract

A sensitive and specific liquid chromatography–tandem mass spectrometry assay was developed to quantify the first selective aldosterone blocker Eplerenone (I) and its hydrolyzed metabolite (II) in human plasma. The analytes (I, II) and their stable isotope-labeled analogues as internal standards were extracted on a C_{18} solid-phase extraction cartridge using a Zymark RapidTrace™ automation system. The chromatographic separation was carried out on a narrow-bore reversed-phase Zorbax XDB-C_8 HPLC column with a mobile phase of acetonitrile/water (40:60, v/v) containing 10 m*M* ammonium acetate (pH 7.4). The analytes were ionized using negative-to-positive switch electrospray mass spectrometry, then detected by multiple reaction monitoring with a tandem mass spectrometer. The precursor to product ion transitions of m/z 415→163 and m/z 431→337 was used to measure I and II, respectively. The assay exhibited a linear dynamic range of 10–2500 ng/ml of plasma for both I and II. The lower limit of quantification was 10 ng/ml for I and II. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. A throughput of 80 human plasma standards and samples per run was achieved with run time of 5 min for each injection. The assay has been successfully used in analyses of human plasma samples to support clinical studies.

Keywords: Eplerenone

1. Introduction

Eplerenone (I), 9,11α-expoxy-17α-hydroxy-3-oxopregn-4-ene-7α, 21-dicarboxylic acid, γ-lactone, methyl ester, a new anti-hypertension drug that is highly selective aldosterone receptor antagonist (SARA) effectively blocks aldosterone at receptor sites in tissues through the body [1,2]. This drug is under clinical development to reduce the rate of all cause mortality in patients who have recently had a heart attack (myocardial infarction, or MI) that resulted in a diagnosis of heart failure. Clinical evidence indicates that aldosterone plays an important role in chronic heart failure, even when other renin–angiotensin–aldosterone system (RAAS) inhibiting agents are employed [3,4]. Moreover, animal studies have indicated that aldosterone, in addition to important renal effects, has direct cardiac and vascular effects [5,6]. These data suggest that an anti-

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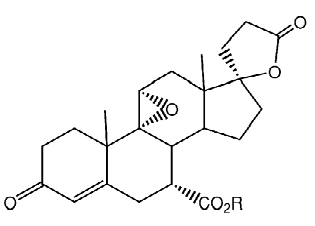
aldosterone therapeutic may provide important protection in chronic heart failure.

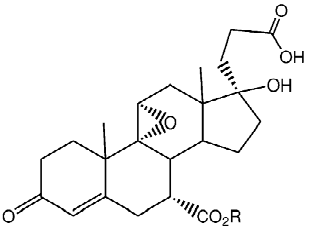
Eplerenone is a steroid nucleus-based antimineralocorticoid that is chemically and enzymatically interconvertible to open lactone form. Under basic conditions, Eplerenone is hydrolyzed to form the open lactone form II (Fig. 1), whereas under acidic conditions, II is converted back to the closed form I. Therefore, it is important to measure both the open and the closed forms of the drug to characterize the pharmacokinetics in man for clinical studies. The challenges for the method development were to prevent the interconversion between the open and closed forms of the drug during sample analyses and to provide sensitive and specific measurement for the drug. Currently, no analytical methods for quantification of Eplerenone and its metabolites have been reported. Several high-performance liquid chromatographic (HPLC) and liquid chromatography–mass spectrometric (LC–MS) methods have been reported in the literature to determine steroids such as spironolactone that is also a γ-lactone in biological fluids [7–11]. However, these methods have not described simultaneous determinations of both the open and closed lactone forms of steroids. Here, we describe the development and validation of a sensitive and specific LC–MS–MS method for the simultaneous quantification of Eplerenone and its hydrolyzed metabolite (II) in human plasma. The procedure consisted of an automated C_{18} solid-phase extraction (SPE) of I, II and their internal standards (III, IV) (Fig. 1) from 0.4 ml of human plasma using a Zymark Rapid Trace automation system. After extraction, the samples were injected onto a reversed-phase Zorbax XDB C_8 HPLC column for separation. I and II were analyzed by tandem mass spectrometry using positive and negative ionization, respectively. The concentrations of I and II were calculated by peak area ratios of the analytes to their internal standards using standard curves generated with weighted linear regression analysis. Accepted precision and accuracy of the assay were achieved for quality controls, plasma hemolysis, three freeze–thaw cycle stability, dilution, long-term stability and sample processing stability. The method is the first validated LC–MS–MS assay to simultaneously monitor Eplerenone and its hydrolyzed metabolite in human plasma and was successfully used to support clinical studies in drug development.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade and were purchased from the following suppliers: methanol and acetonitrile from Baxter Healthcare Co. (Muskegon, MI, USA), ammonium acetate from Aldrich (Milwaukee, WI, USA). High-purity water was obtained using a Millipore system (Milford, MA, USA). I, II, III and IV were synthesized in Pharmacia Incorporation (Skokie, IL, USA) with chemical purity greater than 99%. Human plasma was ac-





R = CH_3 (I, Eplerenone, MW = 414) R = CH_3 (II, hydrolyzed Eplerenone, MW = 432)
R = $^{13}C^2H_3$ (III, MW = 418) R = $^{13}C^2H_3$ (IV, MW = 436)

Fig. 1. Chemical structures of Eplerenone (I), hydrolyzed metabolite (II) and their stable isotope-labeled internal standards (III, IV).

quired from healthy human volunteers who had not taken any medicines for at least 1 month.

2.2. Preparation of standard and quality control samples

Stock solutions of I, II, III and IV (1 mg/ml) were separately prepared in 10-ml volumetric flasks with acetonitrile and water (50:50, v/v). The solutions were serially diluted with water to obtain the desired concentrations. The stock solutions were kept refrigerated (4 °C) and discarded 1 month after preparation. The plasma concentrations of the calibration standards were 10, 50, 100, 500, 1000, 1500, 2000 and 2500 ng/ml. Plasma pools at 10, 100, 500, 1500, 2500 and 10 000 ng/ml were prepared as quality control (QC) and dilution QC samples. These standard and QC samples were prepared with appropriate volumes of I and II stocks in 50-ml volumetric flasks by diluting to the volume with human plasma to achieve the desired concentrations. The freeze–thaw and long-term stability samples were prepared separately for I and II. Then 500-μl aliquots were transferred into 1.8-ml cryotubes (NuncInter Med., Roskilder, Denmark) that were capped and stored in an approximately −20 °C freezer. III and IV internal standard working solution (500 ng/ml) was prepared in a 100-ml volumetric flask by diluting 1 mg/ml stock solutions of III and IV with water.

2.3. Extraction of samples

Frozen human plasma samples were thawed in a water-bath at room temperature. The thawed plasma samples were vortexed and centrifuged at 300 rpm (2000 *g*) at 4 °C for 10 min. Aliquots of 400 μl supernatant from each human plasma standard and validation sample were placed in disposable glass tubes and 400 μl of internal standard aqueous solution (500 ng/ml) was added. Then the plasma samples were vortexed and placed in the loading modules of a RapidTrace™ automatic solid-phase extraction system (Zymark, Hopkinton, MA, USA). C_{18} Bond Elut SPE cartridges (100 mg, 1 cc reservoir, Varian, Harbor City, CA, USA) were conditioned with 2 ml of methanol and 2 ml of water. The plasma samples were loaded onto the cartridges, which were washed with 2 ml of water and eluted with 500 μl of acetonitrile. The solvent was removed under a stream of nitrogen on a TurboVap (Zymark, Hopkinton, MA, USA) at room temperature. The residues were reconstituted in 100 μl of the mobile phase and transferred into autosampler vials. Ten microliters of the reconstituted samples were injected into the LC–MS–MS system for analyses.

2.4. LC–MS–MS

LC–MS–MS analyses were carried out using a system composed of an ISS 200 LC autosampler (Perkin-Elmer, Norwalk, CT, USA), a 1050 HPLC pump (Hewlett-Packard, Wilmington, DE, USA) and an API-III-Plus triplequadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada). The separations were carried out on a narrow-bore reversed-phase Zorbax XDB-C_8 HPLC column (2.1×50 mm, 5 μm, MAC-MOD Analytical, Chadds Ford, PA, USA) with an isocratic mobile phase consisting of acetonitrile/water (40:60, v/v) containing 10 m*M* ammonium acetate (pH 7.4) at a flow-rate of 100 μl/min. The eluate from the HPLC was directly introduced into the mass spectrometer using electrospray ionization in negative-to-positive switch mode with negative ionization for II, IV and positive ionization for I, III, respectively. The electrospray interface and orifice voltages were set at −3600 V and −80 V and at 4400 V and 67 V for negative and positive ionization, respectively. The nitrogen nebulizer gas was set at 60 p.s.i. with the nitrogen curtain gas adjusted to a constant flow-rate of 1.8 l/min. The electrospray interface and mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution. The multiple reaction monitoring (MRM) experiment was conducted by monitoring the precursor ion to product ion transitions from *m/z* 415 (Q1) to *m/z* 163 (Q3) for I and from *m/z* 419 (Q1) to *m/z* 163 (Q3) for its internal standard III, as well as from *m/z* 431 (Q1) to *m/z* 337 (Q3) for II and from *m/z* 435 (Q1) to *m/z* 337 (Q3) for its internal standard IV. The dwell time for each scan was 0.3 s. Argon was used as the collision gas at a gas thickness of 3×10^{15} molecules/cm^2 with colli-

sion offset energy of 25–30 eV to induce fragmentation in the collision cell.

2.5. Method validation

To compile between-run statistics, a plasma calibration curve, a set of validation samples and human plasma blanks to simulate a routine analysis run size of 80 were analyzed on four separate days. For within-run statistics, a plasma calibration curve, five sets of validation samples and human plasma blanks to simulate a routine analysis run size of 80 were analyzed on the fifth day. The first set of validation samples in the within-run experiment was also used for the fifth between-run calculation. The peak areas generated by the MRM of I, II and their internal standards (III, IV) were obtained from the MacQuan data system (PE Sciex, Concord, Ontario, Canada). The ratios of the peak areas of m/z 415→m/z 163 to m/z 419→m/z 163 and m/z 431→m/z 337 to m/z 435→m/z 337 were then calculated for I and II, respectively. Calibration curves were obtained by a weighted $(1/\text{concentration}^2)$ least squares linear regression analysis. Concentrations of I and II in the samples were then calculated using the equations from the appropriate calibration curves. The between-run and within-run precision and accuracy were determined by analyzing five sets of validation samples. The validation criteria of the between-run and within-run precision were 20% or better for the limit of quantification and 15% or better for the rest of concentrations, and the accuracy criteria were 100±20% for the limit of quantification and 100±15% for the rest of concentrations.

3. Results and discussion

3.1. Sample extraction

An automated SPE method was used to isolate and concentrate I and II from human plasma. Performing SPE can be a labor intensive process, especially when large number of samples must be processed, thus the automation allows samples to be processed unattended, often with improved precision and recovery. An eight-module Zymark RapidTrace™ automated extraction system was selected to increase the throughput of the extraction process by reducing the sample preparation time. This system was fully automated and provided high precision and reproducibility. The extraction process included conditioning 100 mg C_{18} Varian Bond Elut SPE columns, loading plasma samples, washing the columns and eluting the analytes from the columns. The total extraction time for 80 plasma samples with these eight modules was approximately 1.5 h.

3.2. Selection of HPLC column and mobile phase

A narrow bore Zorbax XDB-C_8 HPLC column (2.1×50 mm, 5 μm) was used to separate the analytes. The low flow-rate of the narrow bore column was ideal for coupling to an electrospray mass spectrometer. Various mobile phase concentrations of acetonitrile were evaluated to achieve the best separation for I, II, and the internal standards in human plasma. To achieve better ionization in MS, 10 m*M* ammonium acetate was added to the mobile phase. An isocratic system was selected with a mobile phase of acetonitrile/water (40:60, v/v) containing 10 m*M* ammonium acetate (pH 7.4) at a flow-rate of 100 μl/min. Under this condition, the retention times of I and II are approximately 1.3 and 3.3 min, respectively. The stable isotope-labeled internal standards III and IV have the same retention times as I and II. The total run time for each sample was 5 min. Since II and IV are the open lactone forms of II and IV, under acidic conditions I and III are converted into their closed forms I and III, whereas under basic conditions I and III are hydrolyzed to the open forms II and IV. Any interconversion of the open and closed forms will affect accurate measurement of the drugs. In order to prevent the interconversion of the open and closed form, studies were carried out to evaluate the stability of I, II, III and IV. I, II, III and IV were separately added to human plasma and adjusted to different pH conditions. The samples were extracted and analyzed for both I and II or III and IV. The results showed that at pH between 6.5 and 8, I, II, III and IV are stable and no interconversion was observed between the open and closed forms (Fig. 2). Therefore, a neutral pH of mobile phase (pH 7.4) was used during sample separation and analyses.

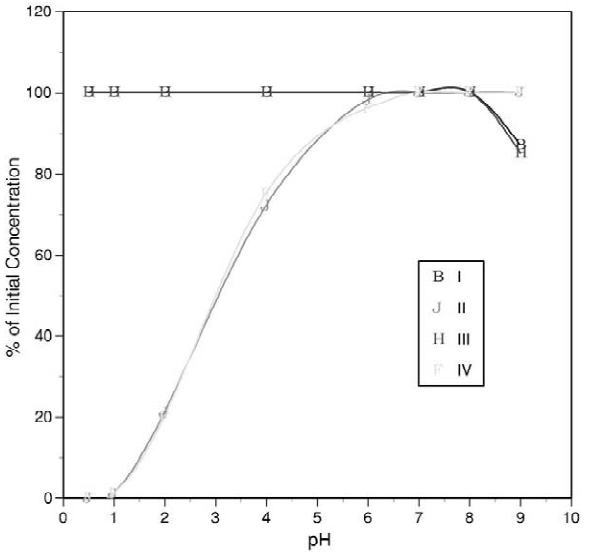


Fig. 2. Stability of I, II, III and IV under different pH conditions. Samples were extracted and analyzed after 0.5 h under different pH conditions.

3.3. Mass spectrometry

Tandem mass spectrometric detection was used to provide a sensitive and selective assay for I and II in human plasma. A negative-to-positive ionization switch mode was used to detect the two analytes in order to achieve the best sensitivity for II and I, respectively. The positive electrospray mass spectrum of I revealed a protonated molecular ion at m/z 415 with the collision-induced dissociation (CID) product ions at m/z 397, 379, 355, 337, 319, 277, 239, 221, 201, 163, 155 and 121 (Fig. 3I). Similarly, its internal standard (III) gave a protonated molecular ion at m/z 419 with the CID product ions at m/z 401, 383, 355, 337, 319, 277, 239, 221, 203, 163, 159 and 121 (Fig. 3III). The negative electrospray mass spectrum of II showed a deprotonated molecular ion at m/z 431 with CID product ions at m/z 381, 355, 337, 299, 289, 283, 225, 212, 175, 123 and 115 (Fig. 3II). Similarly, its internal standard (IV) gave a deprotonated molecular ion at m/z 435 with the same CID product ions as II (Fig. 3IV). MRM mode was used to detect I, II, III and IV by monitoring the precursor ion to product ion transitions of m/z 415$\rightarrow m/z$ 163, m/z 431$\rightarrow m/z$ 337, m/z 419$\rightarrow m/z$ 163, and m/z 435$\rightarrow m/z$ 337, respectively. The representative LC-MRM chromatograms of blank human plasma and 10 ng/ml of I and II with the internal standards are shown in Fig. 4A,B. No significant contribution from blank plasma was observed in the LC-MRM chromatogram, demonstrating the selectivity and specificity of the MRM technique.

3.4. Extraction recovery

Blank human plasma was spiked separately with radiolabeled [^{14}C]-I and [^{14}C]-II at three different concentrations (10, 500 and 2500 ng/ml) in triplicate and extracted by the SPE method as described above. The radioactivity that eluted from the SPE cartridges was compared to the radioactivity spiked in the plasma, and calculated to be 92.5±2.3% ($n=9$) for I and 90.4±2.9% ($n=9$) for II throughout the concentration range.

3.5. Effect of plasma contribution

The matrix contribution at the elution regions of I, II, III and IV in blank male and female human plasma without addition of the analytes was evaluated to ensure the specificity of the method. The mean response for the I peak at the assay sensitivity limit (10 ng/ml) was approximately 7.78- and 5.74-fold greater than the mean response for peaks seen in eight male and female individual blank human plasma samples, respectively, at the retention time of I. The mean response for the II peak at the assay sensitivity limit (10 ng/ml) was approximately 13.7-fold greater than the mean response for peaks seen in eight male individual blank human plasma samples at the retention time of II. There was no response for peaks seen in eight female individual blank human samples at the retention time of II. No response was observed for peaks at the retention time of internal standard III and IV in eight male and six female individual blank human plasma samples. These results demonstrated that no considerable endogenous contribution from human plasma affected the measurement of the analytes.

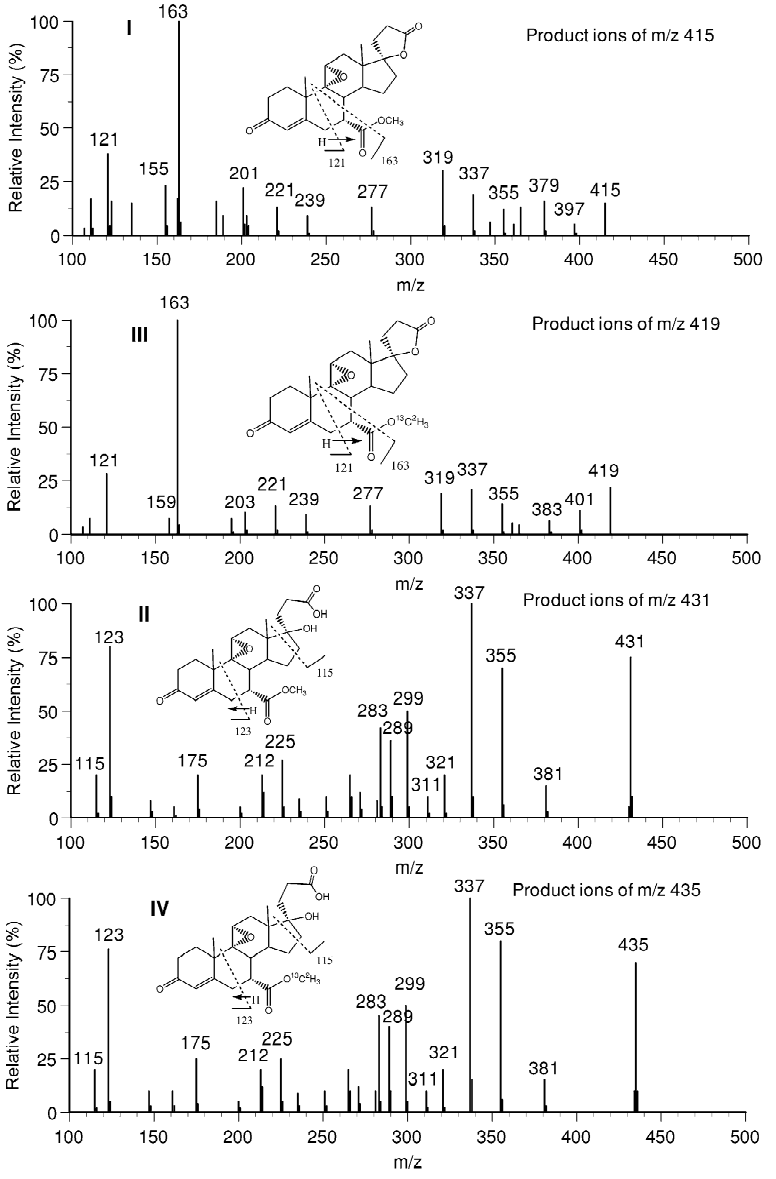


Fig. 3. CID mass spectra of I (m/z 415), III (m/z 419), II (m/z 431) and IV (m/z 435).

3.6. Standard curves

Standard curves were linear over the concentration range of 10–2500 ng/ml for both I and II. A nine-point standard curve gave acceptable results and was used for all the calculations. The mean linear regression equations of standard curves for I and II were $y = 0.00606(\pm 0.00053) + 0.00185(\pm 0.00020)x$ and $y = 0.00439(\pm 0.00035) + 0.00210\ (\pm 0.00017)x$, respectively, where y was the peak area ratio of I to III or II to IV and x was the concentration of I or II. The correlation coefficients of the weighted standard curves generated during the validation ranged from 0.996 to 0.999 for I and 0.996 to 1.00 for II.

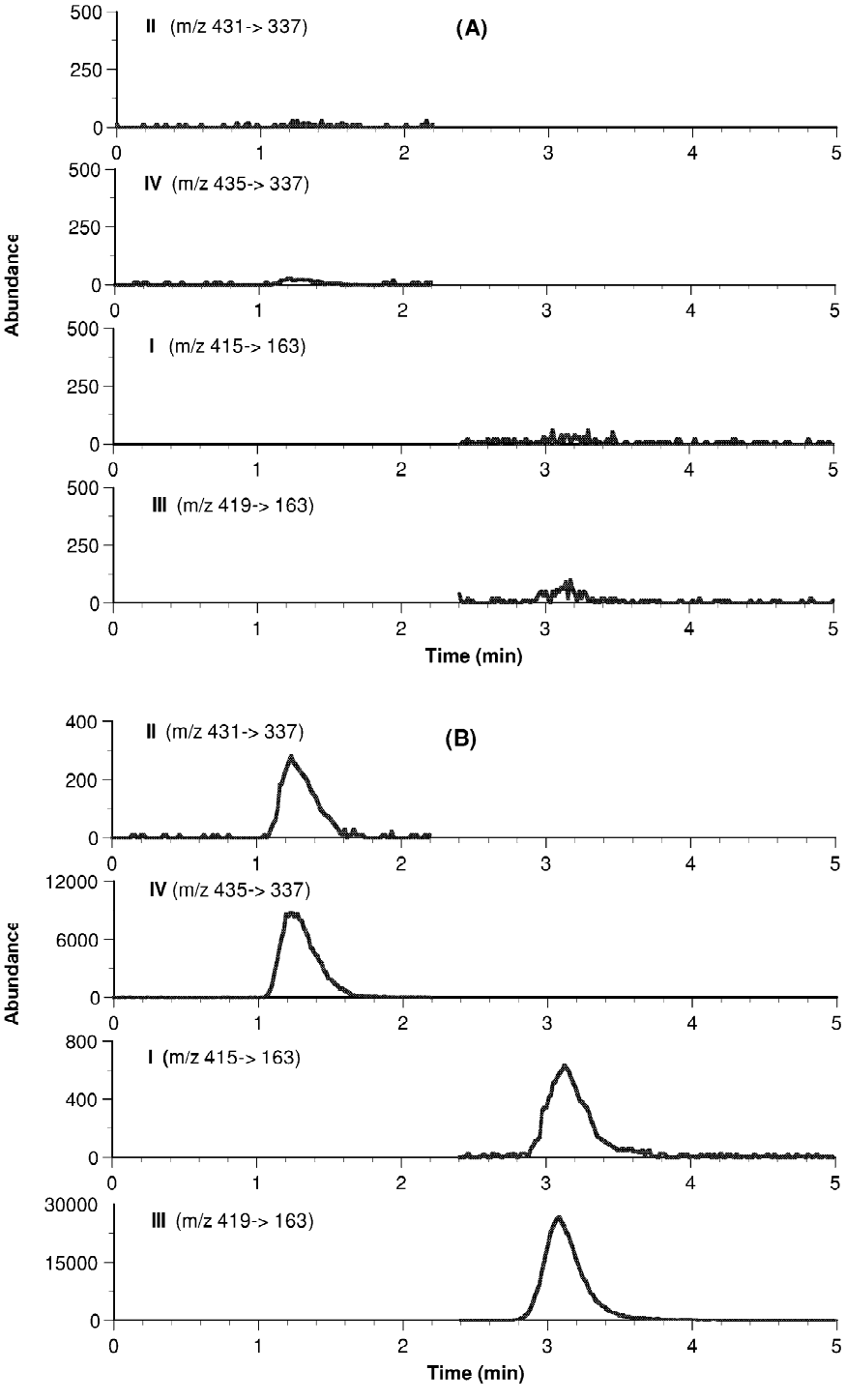


Fig. 4. Representative LC-MRM chromatograms of (A) blank plasma and (B) plasma spiked with both I, II (10 ng/ml) and III, IV (100 ng/ml).

3.7. Lowest concentration

The lower limit of quantification (LLOQ) for the human plasma assay was 10 ng/ml for both I and II. The between-run precision (expressed as coefficient of variation, CV%) was 15.1 and 9.74% for I and II, respectively. The between-run accuracy (expressed as analytical recovery, AR%) was 112 and 99.1% for I and II, respectively (Table 1). The within-run precision was 11.3 and 5.43% and the accuracy was 112 and 99.1% for I and II, respectively (Table 1).

3.8. Middle and upper concentrations

The middle and upper quantification levels ranged from 100 to 2500 ng/ml of I and II in human plasma. For the between-run experiment, precision ranged from 1.53 to 5.85% and the accuracy ranged from 96.0 to 108% for I, and the precision ranged from 2.13 to 4.95% and the accuracy ranged from 95.9 to 102% for II (Table 1). For the within-run experiment, the precision ranged from 1.48 to 5.51% and the accuracy ranged from 96.0 to 108% for I, and the precision ranged from 1.91 to 4.58% and accuracy ranged from 95.9 to 102% for II (Table 1).

3.9. Dilution

The upper concentration limits can be extended with acceptable precision and accuracy to 10 000 ng/ml of I and II by a fivefold dilution with control human plasma. For the between-run experiment, the precision was 4.32 and 3.61% and accuracy was 94.2 and 95.8% for I and II, respectively (Table 1). For the within-run experiment, the precision was 3.49 and 1.72% and accuracy was 94.2 and 95.8% for I and II, respectively (Table 1). Based on these results, samples whose concentrations are greater than the upper limit of the standard curve can be reanalyzed by dilution to obtain acceptable data.

Table 1
Assay validation results obtained from between-run and within-run experiments for I and II in human plasma

Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	Between-run		Within-run	
		CV (%)	AR (%)	CV (%)	AR (%)
Analyte I					
10	11.2	15.1	112	11.3	112
100	108	5.85	108	5.51	108
500	482	2.51	96.4	2.51	96.4
1500	1460	1.53	97.3	1.48	97.3
2500	2400	1.70	96.0	1.64	96.0
10 000[a]	9420	4.32	94.2	3.49	94.2
500_{hemo}[b]	530	3.07	106	2.67	106
$10_{f/t}$[c]	8.90	12.4	89.0		
$20^{c}_{f/t}$	19.8	11.7	99.1		
$500^{c}_{f/t}$	475	2.39	95.0		
Analyte II					
10	9.91	9.74	99.1	5.43	99.1
100	102	4.95	102	4.58	102
500	479	2.13	95.9	2.13	95.9
1500	1487	2.39	99.1	2.23	99.1
2500	2488	3.11	99.5	1.91	99.5
10 000[a]	9580	3.61	95.8	1.72	95.8
500^{b}_{hemo}	530	3.21	106	2.22	106
$10^{c}_{f/t}$	9.28	8.83	92.8		
$20^{c}_{f/t}$	19.1	4.40	95.4		
$500^{c}_{f/t}$	481	3.30	96.1		

[a] The sample was processed with five times dilution.
[b] The sample was from hemolyzed plasma.
[c] The sample was assayed after three freeze–thaw cycles.

3.10. Freeze–thaw stability

The freeze–thaw stability of I and II was determined by measuring the assay precision and accuracy for the samples that underwent three freeze–thaw cycles. The stability data were used to support request for repeat analyses. The frozen plasma samples containing separated I and II were thawed at room temperature for 2–3 h, refrozen for a minimum of 1 day, thawed for 2–3 h, refrozen for a minimum 1 day, thawed and then analyzed. The results showed that I and II were stable in human plasma through three freeze–thaw cycles. The precision ranged from 2.39 to 12.4% and the accuracy ranged from 89.0 to 99.1% for I. The precision ranged from 3.30 to 8.83% and accuracy ranged from 92.8 to 96.1% for II (Table 1). These results suggested that plasma samples could be thawed and refrozen without compromising the integrity of the samples.

3.11. Effect of plasma hemolysis

Hemolyzed human plasma samples containing I and II were evaluated to ensure that the precision and accuracy were acceptable for analysis of hemolyzed plasma samples. The precision was 3.07% and the accuracy was 106% for I, while the precision was 3.21% and the accuracy was 106% for II (Table 1). The results demonstrated that sample hemolysis did not interfere with the assay.

3.12. Assay specificity

To ensure the assay specificity, six human plasma samples from dosed subjects at times approximately equal to C_{max} and two half-lives later, were analyzed using the validated method. Three ion transitions for I and II were monitored for the samples from dosed subjects and for standard solutions. The ion ratios of the ion transitions used in the human plasma assay to the two additional selected ion transitions from the dosed subjects were compared to the ion transition ratios from the standards of I and II. The ion transition ratios of m/z 415→163 to m/z 415→337 and m/z 415→163 to m/z 415→121 for I from the dosed subjects gave an acceptable CV of 10.3 and 20.4%. The relative ratios were 92.9 and 86.7% when compared to the ion transition ratios from the standard (Table 2). The ion transition ratios of m/z 431→337 to m/z 431→355 and m/z 431→337 to m/z 431→299 for II from the dosed subjects gave an acceptable CV with 15.4 and 5.91%. The relative ratios were 102 and 102% when compared to the ion transition ratios from the standard (Table 2). The criteria for the specificity were within 20% for precision and within 100±20% for relative ratios, respectively. These data suggested that no reported metabolites were produced that would interfere with the assay. Also no endogenous substances were produced in response to administration of Eplerenone that would interfere with this assay.

3.13. Processed sample stability

Extracted validation samples at low, middle and high concentrations were kept at room temperature for over 24 h and were then reanalyzed and quantified against freshly made standard curves. The precision and accuracy for I from these extracted and stored samples were 2.44–4.92% and 93.1–98.3%, respectively (Table 3), while the precision and accuracy for II were 1.14–11.8% and 92.2–102%, respectively (Table 3). The results demonstrated that extracted samples could be analyzed after standing at room temperature for at least 24 h with acceptable precision and accuracy.

3.14. Sample collection and processing studies

In vitro studies were carried out to determine whether I and II were lost and/or degraded in human blood during sample collection and processing. In Experiment A, I and II were separately incubated in freshly collected human blood for 15 min on ice followed by centrifugation to separate the plasma from the blood. In Experiment B, I and II were separately incubated in freshly collected human blood for 60 min on ice followed by centrifugation to separate the plasma from the blood. In Experiment C, I and II were separately incubated in freshly collected human blood for 60 min at room temperature followed by centrifugation and leaving the plasma fraction over packed erythrocytes for an additional 60 min at room temperature. Then the plasma was separated by centrifuge and stored in a

Table 2
Assay specificity results for I and II in human plasma

	Ion transition ratios (peak height)	
	(m/z 415→163)/(m/z 415→337)	(m/z 415→163)/(m/z 415→121)
Analyte I		
Standard samples		
Mean (1000 ng/ml, $n=3$)	4.56	7.34
CV (%)	7.45	4.63
Dosed samples		
Mean ($n=6$[a])	4.23	6.36
CV (%)	10.3	20.4
Relative ratio (%)[b]	92.9	86.7
	(m/z 431→337)/(m/z 431→355)	(m/z 431→337)/(m/z 431→299)
Analyte II		
Standard samples		
Mean (1000 ng/ml, $n=3$)	1.01	1.96
CV (%)	2.24	5.53
Dosed samples		
Mean ($n=6$[a])	1.04	1.99
CV (%)	15.4	5.91
Relative ratio (%)[b]	102	102

[a] The samples were from the dosed human plasma at the time points of C_{max} or two half-lives.
[b] Relative ratio (%) = (mean of dose samples/mean of standard samples) × 100.

−20 °C freezer until analysis. The concentration of I from B (incubated on ice for 60 min) and C (incubated at room temperature for 60 min) gave CVs of 2.32 and 3.69% and relative recoveries of 97.6 and 95.8% when compared to the concentrations from control A (incubated on ice for 15 min). Similarly, the concentration of II from B and gave CVs of 2.34 and 1.76% and relative recoveries of 101 and 97.7% when compared to the concentrations from control A. It was thereby concluded that I and II were stable in human blood for at least 60 min at room temperature and in human plasma partitioned over erythrocytes for an additional 60 min at room temperature. The applicability of the devices associated with the collection and processing of human plasma samples containing I and II was studied. An aliquot of the human plasma was passed through a Vacutainer needle during the transfer to a Vacutainer with a green stopper. The concentration of I from E (needle, Vacutainer, green stopper) gave a

Table 3
Processed sample stability results for I and II after 24 h at room temperature

Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	No. of replicates	CV (%)	Recovery (%)
Analyte I				
10	9.31	4	4.92	93.1
500	491	4	2.47	98.3
2500	2420	4	2.44	96.6
Analyte II				
10	9.22	4	11.8	92.2
500	489	4	1.14	97.8
2500	2548	4	2.47	102

CV of 0.353% and a relative recovery of 99.3% when compared to the concentration from the control sample D (no collection device). Similarly, the concentration of II from E gave a CV of 1.06% and a relative recovery of 101% when compared to the concentration from the control sample D. These data suggested that the procedure used in the collection to obtain the blood samples should not introduce any bias in the samples.

3.15. Long-term storage stability

The sample long-term storage stability at −20 °C was evaluated to establish acceptable storage conditions for clinical samples. Parts of human plasma samples spiked separately with I and II at concentrations of 10 and 500 ng/ml were analyzed on Day 1. Then the samples from the same pools were analyzed after storage at −20 °C for 90 days against freshly made standard curves. The CV and AR for I on Day 90 ranged from 1.95 to 3.90% and 96.0 to 96.8%, respectively. For II on Day 90, the CV ranged from 2.57 to 4.68% and the AR ranged from 99.2 to 108% (Table 4). The results indicated that I and II were stable when stored frozen at −20 °C for at least 90 days.

3.16. Clinical application

The validated method was applied to two clinical studies with over 2200 human plasma samples analyzed in 30 analytical runs. Approximately 120 human plasma samples were analyzed along with standards and QCs in two runs per day. For each run, six QCs (duplicated at three concentration levels) and two dilution QCs (when dilution samples were assayed) were randomly located among the clinical samples within a run. Approximately 5% of the analyzed samples exceeded the upper concentration of the analytes I and were reanalyzed after fivefold dilution. I and II could be detected for up to 16 and 8 h, respectively, after a single oral 100-mg dose of Eplerenone. The mean I and II human plasma concentrations after a 100-mg dose of Eplerenone to eight human volunteers are shown in Fig. 5. The maximum concentrations (C_{max}) of I and II in plasma were 1721.3±289.8 and 82.8±17.2 ng/ml, respectively. The areas under the plasma concentration/time curve (AUC) were 9527±3201 and 352.2±115.1 ng h/ml, respectively. These results indicate the successful application of this method in clinical studies.

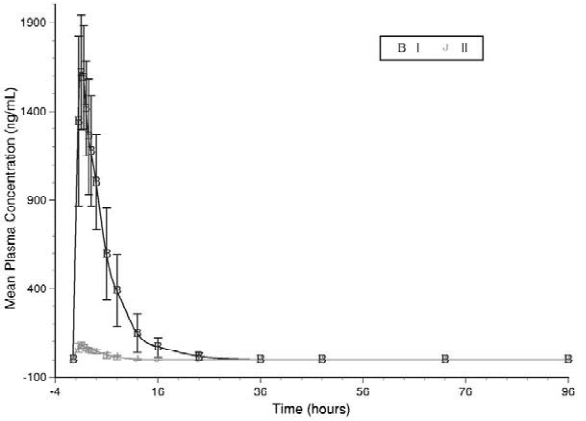


Fig. 5. Mean I and II human plasma concentrations (ng/ml) following a 100-mg dose of Eplerenone.

Table 4
Results for long-term frozen storage stability samples for I and II at −20 °C

Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	Storage (days)	No. of replicates	CV (%)	Relative recovery (%)
Analyte I					
10	9.60	90	3	3.90	96.0
500	484	90	3	1.95	96.8
Analyte II					
10	10.8	90	3	4.68	108
500	496	90	3	2.57	99.2

4. Conclusions

An automated, high throughput and sensitive LC–MS–MS assay was developed to support clinical pharmacokinetic studies in Eplerenone drug development. The assay was demonstrated for the simultaneous determination of Eplerenone and its hydrolyzed metabolite in human plasma. Tandem mass spectrometric detection was used to provide a sensitive and selective assay. A negative-to-positive ionization switch mode was used to detect the two analytes in order to achieve the best sensitivity. Acceptable data were generated from both Eplerenone and its hydrolyzed metabolite using a weighted linear regression ($1/\text{concentration}^2$) and full standard curves for human plasma samples. The LLOQ was 10 ng/ml for both analytes. Acceptable precision and accuracy were obtained for concentrations above the sensitivity limit and within the standard curve range of 10–2500 ng/ml. The upper concentration limit can be extended, with acceptable precision and accuracy, to 10 000 ng/ml by a fivefold dilution with blank human plasma. Hemolyzed human plasma can be analyzed with acceptable precision and accuracy. Collection devices for use in the collection of human blood were also evaluated and proved to be acceptable. Eplerenone and its hydrolyzed metabolite were stable for at least 60 min at room temperature in human blood and an additional 60 min at room temperature in plasma over packed erythrocytes. Extracts of Eplerenone and its hydrolyzed metabolite can be analyzed after standing at room temperature for at least 24 h with acceptable precision and accuracy. The validated SPE-LC–MS–MS human plasma assay was specific for Eplerenone and its hydrolyzed metabolite in human plasma and the samples can be stored frozen at $-20\,^{\circ}\text{C}$ for at least 90 days. The 5-min total run time made it possible to analyze 80 samples per run in 8 h and 160 samples with two analytical runs per day. The method was sensitive enough to permit pharmacokinetic studies of Eplerenone disposition after oral administration of the drug to humans and was successfully applied for the determination of Eplerenone and its hydrolyzed metabolite in human plasma for clinical studies.

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