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A validated SPE–LC-MS/MS assay for Eplerenone and its hydrolyzed metabolite in human urine

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Abstract

An automated LC-MS/MS assay was validated to quantitate the first selective aldosterone blocker Eplerenone (I) and its hydrolyzed metabolite (II) in human urine. After the addition of the stable isotope labeled internal standards, human urine samples were extracted on a C_{18} solid phase extraction (SPE) cartridge using a Zymark RapidTrace™ automation system. The extraction eluates were diluted with 20 mM ammonium acetate aqueous solution and directly injected onto the LC-MS/MS system. The chromatographic separation was performed on a reverse phase Zorbax XDB-C_8 HPLC column (2.1 × 50 mm, 5 μm) with a mobile phase of acetonitrile:water (40:60, v/v) containing 10 mM ammonium acetate (pH 7.4). I and II were ionized using positive and negative ionization mass spectrometry, respectively, to achieve the best sensitivity. The ionization polarity was switched during the run at approximately 2.5 min after the injection. Multiple reaction monitoring (MRM) with a tandem mass spectrometer was used to detect the analytes. The precursor to product ion transitions of m/z 415 → 163 and m/z 431 → 337 were used to measure I and II, respectively. The assay exhibited a linear dynamic range of 50–10 000 ng/ml of urine for both of I and II. The lower limit of quantitation (LLOQ) was 50 ng/ml for I and II. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. Sample analysis time for each injection was 5 min; a throughput of 100 human urine standards and samples per run was achieved.

Keywords: LC-MS/MS; Solid phase extraction; Eplerenone; Selective aldosterone receptor antagonist (SARA)

1. Introduction

Eplerenone (I) is the first highly selective aldosterone receptor antagonist (SARA) to effectively block aldosterone at receptor sites in body tissues [1–3]. This drug is under clinical development for the treatment of hypertension and heart failure. Clinical studies demonstrate that aldosterone plays an important role in chronic heart failure, even when other renin–angiotensin–aldosterone system (RAAS) inhibiting agents are employed [4–6]. New pre-clinical data generated over the past few years suggest that aldosterone, in addition to important renal effects, has direct cardiac and vascular effects [7,8]. Eplerenone is a steroid nucleus-based antimineralocorticoid that is

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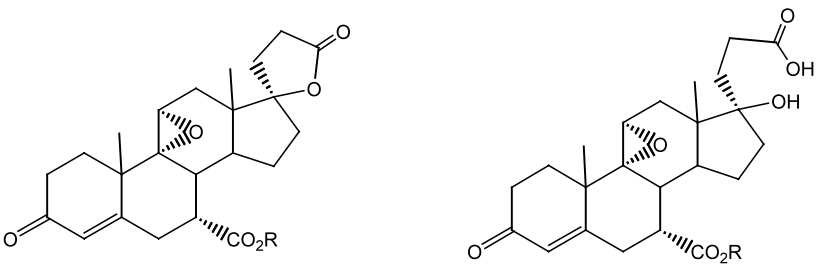

R= CH_3 (I, Eplerenone)
R= $^{13}C^2H_3$ (III)

R= CH_3 (II, hydrolyzed Eplerenone)
R= $^{13}C^2H_3$ (IV)

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

chemically and enzymatically interconvertible to an open lactone ring 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 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.

To date no analytical methods for determination of Eplerenone have been reported. Methods of HPLC with UV detection have been developed to measure a closely related compound, spironolactone, in biological fluids [9–11]. However, lack of specificity of drug and metabolites and long run times of these assays do not meet modern drug development needs, which require a rapid feedback of analytical information from pre-clinical and clinical pharmacokinetics studies in order to compete in the market conditions today. Liquid chromatograph (LC)-tandem mass spectrometry (MS/MS) is widely used in determination of drugs including some steroids in biological fluids [12–15]. The technique provides some advantages such as high-throughput, sensitivity and specificity that are needed for the assay development for Eplerenone. This paper describes the development and validation of a sensitive and specific LC-MS/MS method for the simultaneous quantitation of Eplerenone and its hydrolyzed metabolite (II) in human urine. The procedure consisted of an automated C_{18} solid phase extraction (SPE) of I, II and their internal standards (III, IV) (Fig. 1) from 0.5 ml of human urine using a Zymark Rapid Trace automation system. After extraction, the samples were diluted and directly injected onto a 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. Precision and accuracy of the assay were evaluated for quality controls (QCs), 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 determine the concentrations of Eplerenone and its hydrolyzed metabolite in human urine and was successfully used to support clinical studies in drug development.

2. Experimental

2.1. Chemicals and reagents

Compound I, II, III and IV were synthesized at Pharmacia Corporation (Skokie, IL). All chemicals were of analytical grade and were purchased from the following suppliers: acetonitrile from

Baxter Healthcare Co. (Muskegon, MI), ammonium acetate from Aldrich Chemical Co., (Milwaukee, WI). High-purity water was obtained using a Millipore system (Milford, MA). Human urine blank were collected from healthy human volunteers.

2.2. Preparation of standard and quality control samples

Stock solution 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 urine concentrations of the calibration standards were 50, 100, 250, 500, 1000, 2500, 5000, 7500 and 10 000 ng/ml. Urine pools at 50, 100, 1000, 10 000, and 50 000 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 urine to achieve the desired concentrations. Then 750 μl of aliquots were transferred into 1.8 ml cryotubes (NuncInter Med, Roskilder, Denmark) that were capped and stored in an approximately −70 °C freezer. III and IV internal standard solution (1 μg/ml) was prepared in a 100 ml volumetric flask by diluting 1 mg/ml stock solutions of III and IV with 20 mM ammonium acetate aqueous buffer (pH 7.4).

2.3. Extraction of samples

Human urine standard and validation samples were thawed in a water bath at room temperature and then centrifuged at approximately 2000 g at 4 °C for 5 min. Aliquots of 500 μl from each sample were transferred to disposable glass tubes and 500 μl of the internal standard solution (1 μg/ml) was added. For the dilution samples, 50 μl of the urine samples was diluted with 450 μl of human urine blank before the addition of the internal standards. The urine samples were vortexed and placed in the loading modules of a RapidTrace™ automatic SPE system (Zymark, Hopkinton, MA). C_{18} Bond Elut SPE cartridges (100 mg, 1 cc reservoir, Varian, Harbor City, CA) were conditioned with 2 ml of acetonitrile and 2 ml of water. The urine samples were loaded onto the cartridges, which were washed with 3 ml of water and eluted with 250 μl of acetonitrile. The eluates were diluted with 250 μl of 20 mM ammonium acetate buffer (pH 7.4), vortexed and transferred into autosampler vials. Twenty microliter of the samples were injected into the LC-MS/MS system for analyses.

2.4. LC-MS/MS

LC-MS/MS analyses were performed using a system comprised of an ISS 200 LC autosampler (Perkin–Elmer, Norwalk, CT), a 1050 HPLC pump (Hewlett–Packard, Wilmington, DE) and an API-III Plus quadrupole mass spectrometer (PE Sciex, Concord, Ontario). The separations were carried out on a Zorbax XDB-C_8 HPLC column (2.1 × 50 mm, 5 μm, MAC-MOD Analytical, Chadds Ford, PA) with an isocratic mobile phase consisting of acetonitrile:water (40:60, v/v) containing 10 mM 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 ionspray ionization in negative-to-positive switch mode with negative ionization for II, IV and positive ionization for I, III, respectively. The ionspray interface and orifice voltages were set at −3600 and −80 and at 4400 and 67 V for negative and positive ionization, respectively. The nitrogen nebulizer gas was set at 60 psi with the nitrogen curtain gas adjusted to a constant flow rate of 1.8 l/min. The ionspray 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. Argon was used as the collision gas at gas thickness of 3×10^{15} molecules

per cm^2 with collision offset energy of 25–30 eV to induce fragmentation in the collision cell.

2.5. Method validation

To compile between-run statistics, a urine calibration curve, a set of validation samples and human urine blanks from five male and five female volunteers to simulate a routine analysis run size of 100 were analyzed on four separate days. For within-run statistics, a urine calibration curve, five sets of validation samples and human urine blanks to simulate a routine analysis run size of 100 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 standard (III, IV) were obtained from the MacQuan data system (PE Sciex, Concord, Ontario). The ratios of the peak areas of m/z 415 $\rightarrow m/z$ 163 to m/z 419 $\rightarrow m/z$ 163 and m/z 431 $\rightarrow m/z$ 337 to m/z 435 $\rightarrow m/z$ 337 were then calculated for I and II, respectively. Calibration curves were obtained by a weighted (1/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 was 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 quantitation and 15% or better for the rest of concentrations, and the accuracy criteria were $100 \pm 20\%$ for the limit of quantitation and $100 \pm 15\%$ for the rest of concentrations. The contributions from five male and five female human urine blanks were evaluated to determine matrix effects and subject to subject variability.

3. Results and discussion

3.1. Method development

An automated SPE method was used to isolate and concentrate I and II from human urine. 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. A ten-module Zymark RapidTrace^{TM} 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 urine samples, washing the columns and eluting the analytes from the columns. The total extraction time for 100 urine samples with these ten modules was approximately 1.5 h.

A 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 ionspray mass spectrometer. Various mobile phase concentrations of acetonitrile and ammonium acetate buffer were evaluated to achieve the best separation for I, II and the internal standards in human urine. An isocratic system was selected with a mobile phase of acetonitrile:water (40:60, v/v) containing 10 mM 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 I and II, under acidic conditions II and IV 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 performed to evaluate the stability of I, II, III and IV.Compound I, II, III and IV were separately added into human urine 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 under neutral pH conditions I, II, III and IV are stable and no interconversion was observed be-

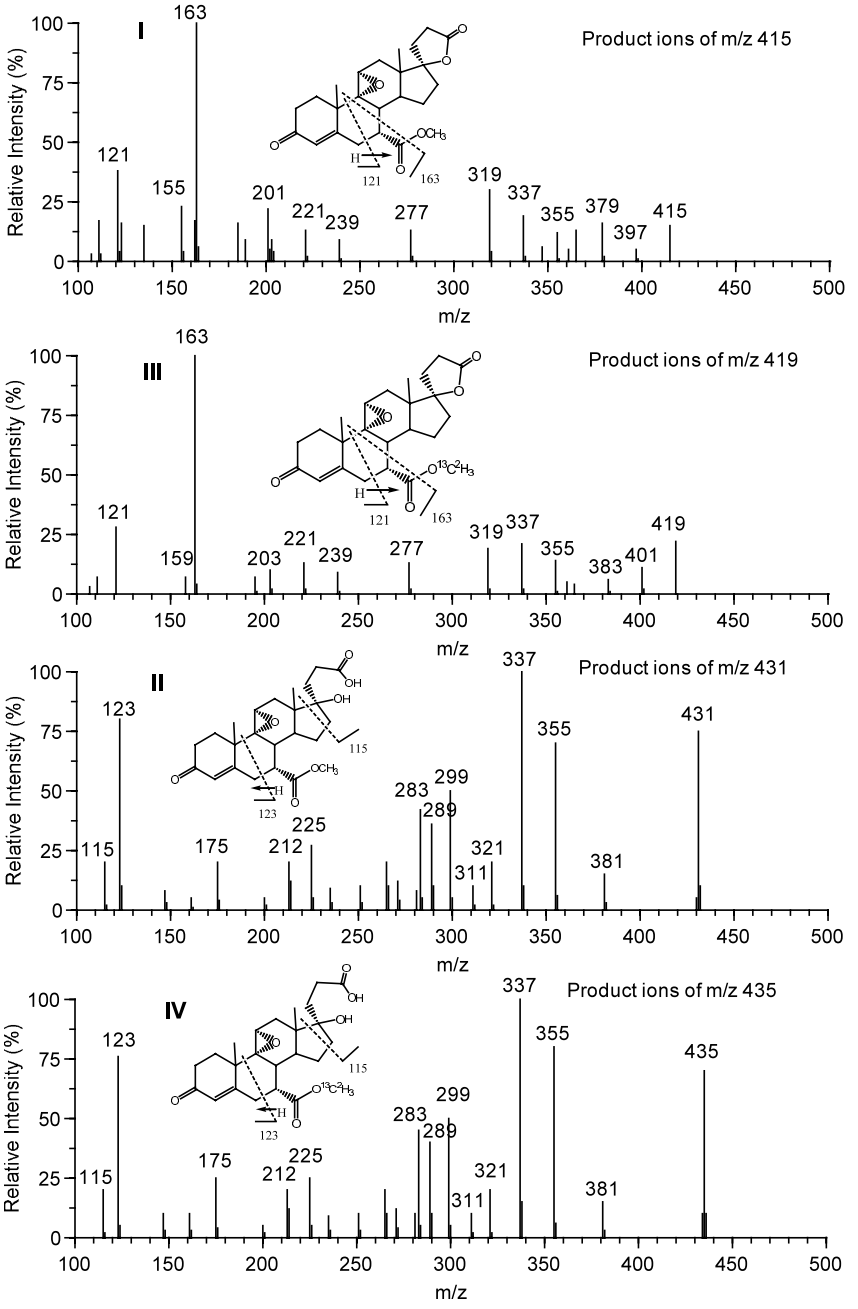

Fig. 2. The CID spectra of I (m/z 415), II (m/z 431), III (m/z 419), and IV (m/z 435).

tween the open and closed forms. Therefore, neutral pH mobile phase and buffer (pH 7.4) were used during sample separation and analyses.

Tandem mass spectrometric detection was used to provide a sensitive and selective assay for I and II in human urine. A negative-to-positive ioniza-

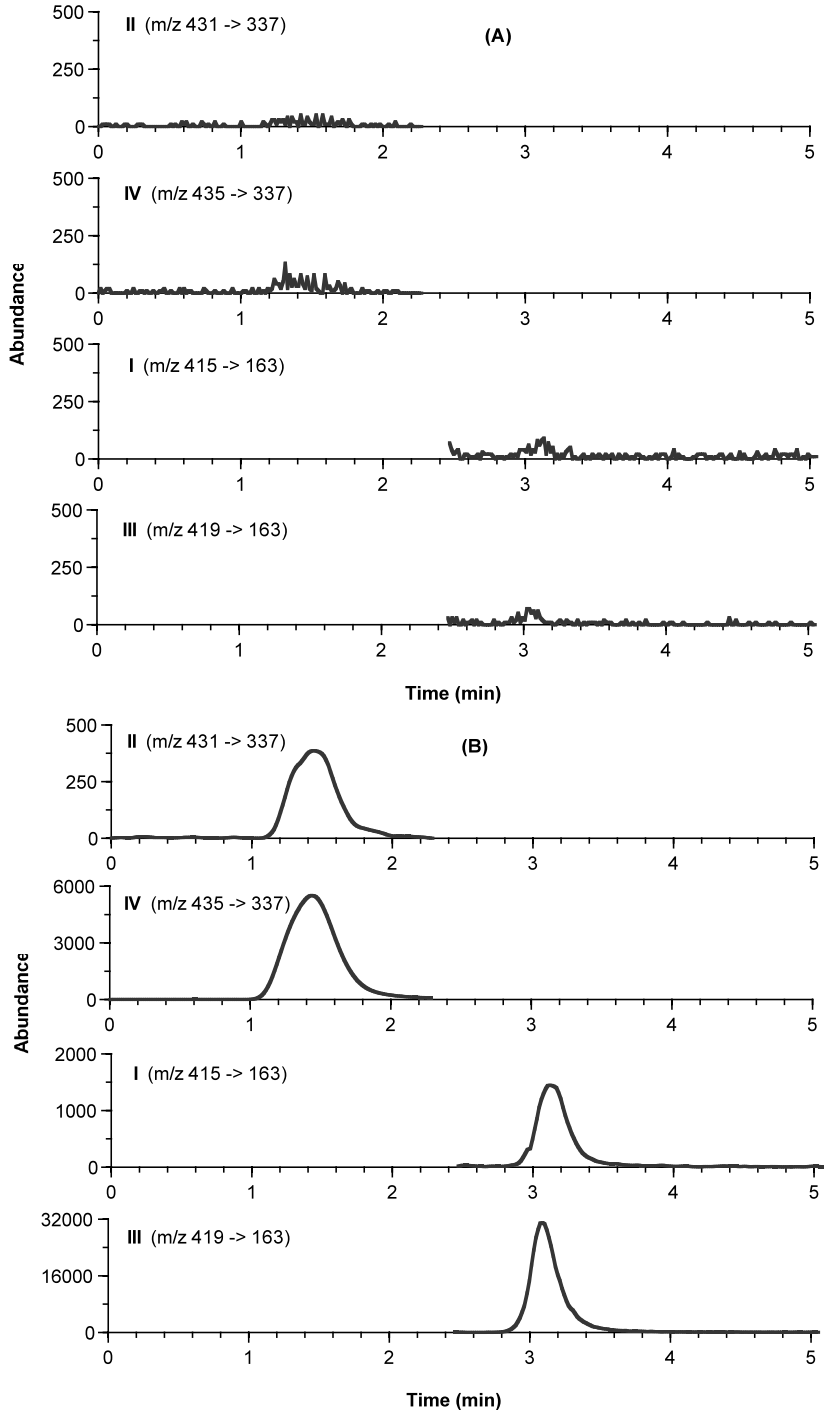

Fig. 3. Representative LC-MRM chromatograms of (A) human urine blank and (B) human urine spiked with I, II (50 ng/ml) and III, IV (1 μg/ml).

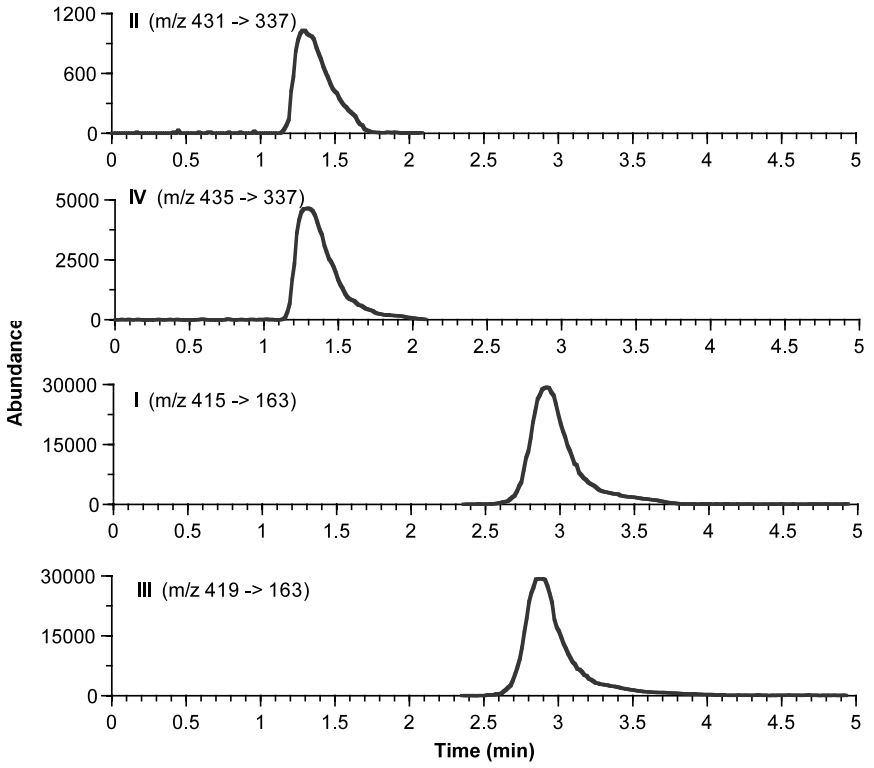

Fig. 4. Representative LC-MRM chromatograms of the human urine sample from a human subject after a dose of 100 mg of Eplerenone.

tion switch mode was used to detect the two analytes in order to achieve the best sensitivity for II and I, respectively. The positive ionspray 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. 2I). 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. 2III). The sequential loss of two waters from the *m/z* 415 (I) and the *m/z* 419 (III) formed the product ions at *m/z* 397, 379 and *m/z* 401, 383, respectively. The product ion at *m/z* 355 was generated from the loss of $HCOOCH_3$ or $HCOO^{13}C^2H_3$ from the *m/z* 415 or the *m/z* 419, respectively. The further loss of two waters from the *m/z* 355 resulted in the product ions at *m/z* 337 and 319. The base peak at *m/z* 163 was formed as illustrated in Fig. 2I and III, which lost $COCH_2$ to yield the product ion at *m/z* 121. The negative ionspray 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. 2II). Similarly, its internal standard (IV) gave a deprotonated molecular ion at *m/z* 435 with the same CID product ions as II (Fig. 2IV). The major product ions at *m/z* 355 and 337 arose from the sequential loss of $HO+COOCH_3$ (or $COO^{13}C^2H_3$) and water from the *m/z* 431 (or *m/z* 435). The product ions at *m/z* 123 and 115 corresponded the fragments as illustrated in Fig. 2II, IV. MRM mode was used to detect I, II, III and IV by monitoring the precursor ion to product ion transitions of *m/z* 415 → *m/z* 163, *m/z* 431 → *m/z* 337, *m/z* 419 → *m/z* 163, and *m/z* 435 → *m/z* 337, respectively. The negative to positive ionization polarity was switched during the run at approximately 2.5 min after the injection. The

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

Spiked concentration (ng/ml)	Number of replicates	Between-run		Within-run	
		CV (%)	AR (%)	CV(%)	AR (%)
Analyte I					
50	9	7.95	98.5	8.55	99.2
100	9	2.46	101	5.26	97.6
1000	9	1.65	102	2.13	105
10 000	9	2.96	95.8	2.23	103
50 000[a]	9	5.34	97.7	3.49	101
$100_{f/t}$[b]	5	8.32	113		
$1000_{f/t}$[b]	5	2.11	99.8		
Analyte II					
50	9	7.54	96.6	5.43	102
100	9	4.33	94.9	4.58	99.4
1000	9	2.06	101	2.23	98.2
10 000	9	2.51	101	1.91	104
50 000[a]	9	6.16	102	1.72	100
$100_{f/t}$[b]	5	7.99	102		
$1000_{f/t}$[b]	5	1.09	100		

[a] The sample was processed with 10-fold dilution.
[b] The sample was assayed after three freeze–thaw cycles.

LC-MRM chromatograms of blank human urine and 50 ng/ml of I and II with the internal standards demonstrated the selectivity and specificity of the MRM technique (Fig. 3 A and B).

3.2. Extraction recovery

Blank human urine was spiked separately with radiolabeled [^{14}C]-I and [^{14}C]-II at two different concentrations (10 and 500 ng/ml) in triplicate and extracted by the SPE method described above. The radioactivity that eluted from the SPE cartridges was compared with the radioactivity spiked in the urine. The extraction recoveries for I and II were 82.0% ($n = 6$) and 89.3% ($n = 6$), respectively.

3.3. Validation

3.3.1. Standard curves

Standard curves were linear over the concentration range of 50–10 000 ng/ml for both I and II. Nine point standard curves gave acceptable results and were used for all the calculations (Fig. 5). The correlation coefficients of the weighted standard curves generated during the validation ranged from 0.997 to 1.00 for I and 0.996 to 0.998 for II. The standard curves obtained as described above were suitable for generation of acceptable data for the concentrations of I and II in samples from between-run and within-run validations.

The validation samples were randomized daily, processed and analyzed in positions either (a) immediately following the standard curve, (b) in the middle of the run, or (c) at the end of the run. The data are summarized in Table 1.

3.3.2. Lowest concentration

The lower limit of quantitation (LLOQ) for the human urine assay was 50 ng/ml for both I and II. The between-run precision (expressed as coefficient of variation, CV%) was 7.95 and 7.54% for I and II, respectively. The between-run accuracy (expressed as analytical recovery, AR%) was 98.5 and 96.6% for I and II, respectively (Table 1). The within-run precision was 8.55 and 5.43% and the accuracy was 99.2 and 102% for I and II, respectively (Table 1).

3.3.3. Middle and upper concentrations

The middle and upper quantitation levels ranged from 100 to 10 000 ng/ml of I and II in human urine. For the between-run experiment, precision ranged from 1.65 to 2.96% and the accuracy ranged from, 95.8 to 102% for I, and the precision ranged from 2.06 to 4.33% and the accuracy ranged from 94.9 to 101% for II (Table 1). For the within-run experiment, the precision ranged from 2.13 to 5.26% and the accuracy ranged from 97.6 to 105% for I, and the precision ranged from 1.91 to 4.58% and accuracy ranged from 98.2 to 104% for II (Table 1).

3.3.4. Dilution

The upper concentration limits can be extended with acceptable precision and accuracy to 50 μg/ml of I and II by a 10-fold dilution with human urine blank. For the between-run experiment, the precision was 5.34 and 6.16% and accuracy was 97.7 and 102% for I and II, respectively (Table 1). For the within-run experiment, the precision was 3.49 and 1.72% and accuracy was 101 and 100% for I and II, respectively (Table 1). Thus, samples whose concentrations are greater than the upper limit of the standard curve can be calculated to obtain acceptable data.

3.3.5. 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 repeat analyses. The frozen urine samples containing separated I and II were thawed at room temperature for 2–3 h, refrozen for minimum of 1 day, thawed for 2–3 h, refrozen for minimum 1 day, thawed and then analyzed. The results showed that I and II were stable in human urine through three freeze–thaw cycles. The precision ranged from 2.11 to 8.32% and the accuracy ranged from 99.8 to 113% for I. The precision ranged from 1.09 to 7.99% and accuracy ranged from 100 to 102% for II (Table 1). These results suggested that urine samples can be thawed and refrozen without compromising the integrity of the samples.

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

	Ion transition ratios (peak height)	
Analyte I	(m/z 415 → 163)/(m/z 415 → 337)	(m/z 415 → 163)/(m/z 415 → 121)
Standard samples		
Mean (1000 ng/ml, $n = 3$)	4.45	6.88
CV (%)	8.54	6.32
Dosed samples		
Mean ($n = 6$)	4.55	6.69
CV (%)	9.32	12.8
Relative ratio (%)[a]	102	97.2
Analyte II	(m/z 431 → 337)/(m/z 431 → 355)	(m/z 431 → 337)/(m/z 431 → 299)
Standard samples		
Mean (1000 ng/ml, $n = 3$)	1.08	1.92
CV (%)	3.44	4.56
Dosed samples		
Mean ($n = 6$)	1.02	1.95
CV (%)	12.8	9.44
Relative ratio (%)[a]	94.4	102

[a] Relative ratio (%) = (mean of dose samples/mean of standard samples) × 100.

3.4. Effect of matrix

The blank contribution at the elution regions of I, II, III and IV in control male and female human urine without addition of the analytes was evaluated to ensure the specificity of the method. The mean response at the retention times of I, II, III and IV in five male and female individual human urine blanks was at least ten times less than the response at the assay sensitivity limit (50 ng/ml). There was no significant subject to subject variability in their LC-MS/MS chromatograms from five male and female individual human urine. A typical LC-MS/MS chromatogram is shown in Fig. 3A. These results demonstrated that no considerable endogenous contribution from human urine blanks affects the measurement of the analytes.

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

Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	Number of replicates	CV (%)	Recovery (%)
Analyte I				
100	98.3	4	5.92	98.3
1000	991	4	6.45	99.1
Analyte II				
100	92.6	4	10.8	92.6
1000	989	4	3.55	98.9

Table 4
Results for long-term frozen storage stability samples for I and II at −70 °C

Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	Storage days	Number of replicates	CV (%)	Relative recovery (%)[a]
Analyte I					
100	110	37	3	5.85	110
1000	1020	37	3	1.89	102
Analyte II					
100	98.2	37	3	7.88	98.2
1000	1030	37	3	6.01	103

[a] Relative ratio (%) = (mean concentration on day 37/mean concentration on day 1) × 100.

3.5. Assay specificity

To ensure that the assay was specific for I and II in human urine, six human urine samples from dosed subjects at 4–8 and 8–12 h postdose 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 urine assay to the two additional selected ion transitions from the dosed subjects were compared with 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 9.32 and 12.8%. The relative ratios were 102 and 97.2% when compared with 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 12.8 and 9.44%. The relative ratios were 94.4 and 102% when compared with 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.

3.6. Processed sample stability

Extracted validation samples at two different concentrations were kept at room temperature for over 24 h and were then reanalyzed and quantitated against freshly made standard curves. The precision and accuracy for I from these extracted and stored samples were 5.92–6.45 and 98.3–99.1%, respectively (Table 3), while, the precision and accuracy for II were 3.55–10.8 and 92.6–98.9%, respectively (Table 3). The results demonstrated that extracted samples could be analyzed after standing at room temperature for at least 24

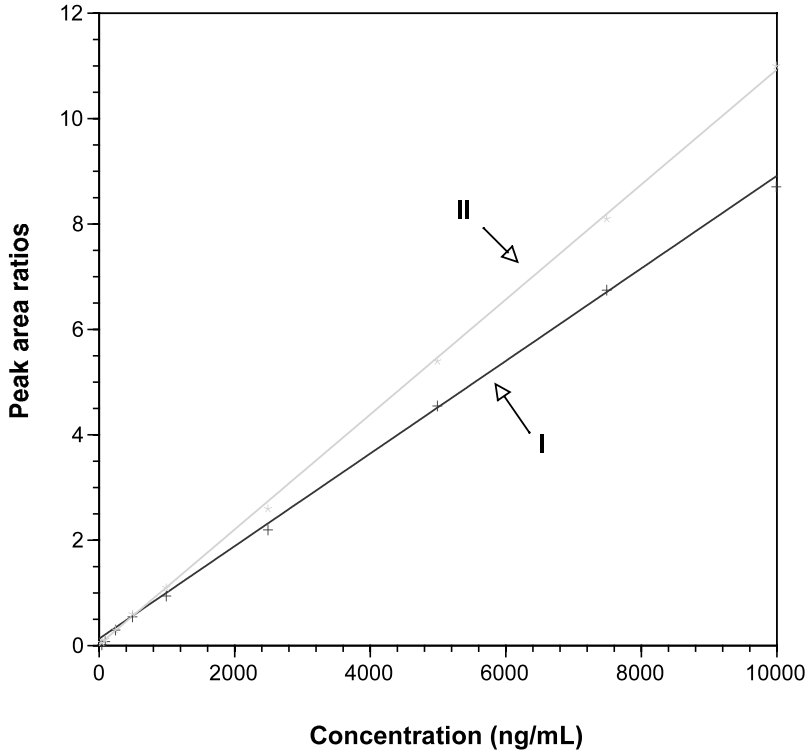

Fig. 5. Representative standard curves of I and II in human urine in the range of 50–10 000 ng/ml.

h with acceptable precision and accuracy. Therefore, the extracted samples can be reinjected for repeat analyses within 24 h in the event of an instrument failure.

3.7. Long-term storage stability

The sample long-term storage stability at −70 °C was evaluated to establish acceptable storage conditions for clinical samples. Aliquots of human urine samples spiked separately with I and II at concentrations of 100 and 1000 ng/ml were analyzed on day 1. Then the samples from the same pools were analyzed after storage at −70 °C for 37 days using freshly made standard curves. The results indicated that I and II were stable when stored frozen at −70 °C for at least 37 days. The CV for I and II on day 37 ranged from 1.89 to 5.85 and 6.01 to 7.88%, respectively. The relative recoveries for I and II on day 37 compared with these on day 1 ranged from 102 to 110 and 98.2 to 103%, respectively (Table 4).

3.8. Length of run

A run was defined as a group of standards, validation and blank samples that were processed through automated SPE, analyzed by serial LC-MS/MS injections and calculated from standard curves the in the group. A total of 100 human urine standards and samples can be analyzed per run with acceptable precision and accuracy.

3.9. Clinical application

This method was applied to two clinical studies with over 1200 human urine samples from ten human subjects analyzed in 14 analytical runs. Approximately 160 human urine samples were analyzed along with standards and QCs in two runs per day. For each run, six QCs (duplicated at

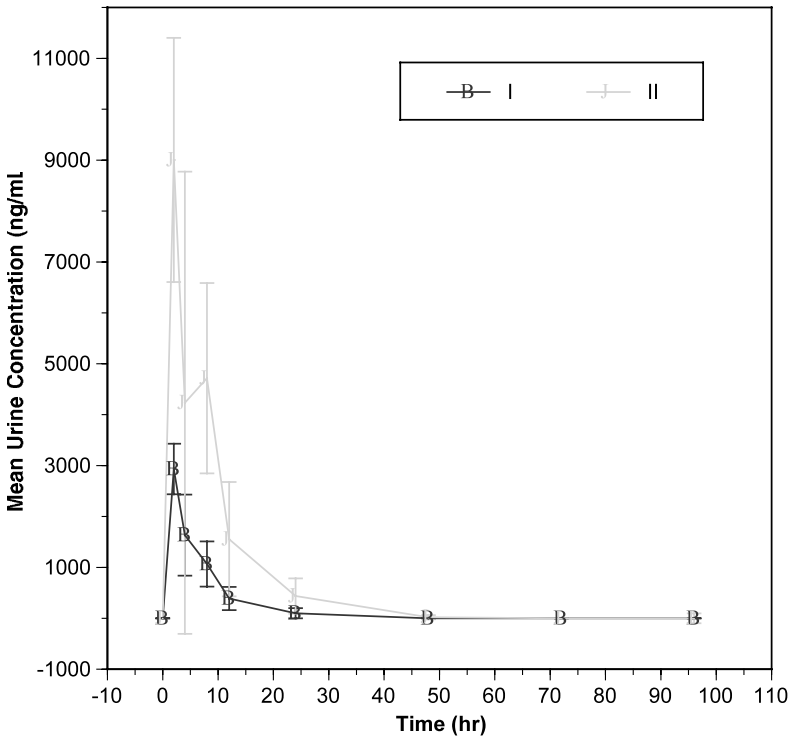

Fig. 6. Mean I and II urine concentration–time profiles following a 100 mg oral dose of Eplerenone to human subjects. Bars represent standard deviations.

three concentration levels) and two dilution QCs (when dilution samples were assayed) were randomly located among the clinical samples within a run. Approximately 8% of the analyzed samples exceeded the upper concentration of the standard curves and were reanalyzed by 10-fold dilution. All the samples were blinded during the runs. No interference peak was found in predose −12–0 h human urines for all ten human subjects. All the metabolites of Eplerenone were identified in human urine (C. Cook unpublished works) and were confirmed to not interfere with the assay. The precision and accuracy of QCs from these studies met the validation criteria ($<20\%$ and $<100\pm 20\%$ for LLOQ, <15 and $<100\pm 15\%$ for the rest of concentrations). Typical LC-MS/MS chromatograms from human urine after a 100 mg dose of Eplerenone are shown in Fig. 4. The mean I and II urine concentration–time profile following a 100 mg oral dose of Eplerenone to human subjects is shown in Fig. 6. No detectable levels of I and II were found in the urine after 24 and 48 h postdose, respectively. On average, the maximum amounts excreted as I and II were during the 2–4 and the 4–8 h postdose collection periods, respectively. The pharmacokinetic parameters obtained from these studies will be reported in detail elsewhere.

4. Conclusions

We have developed and validated an automated, high throughput and sensitive LC-MS/MS assay to support clinical studies in Eplerenone drug development. The assay was the first method developed to simultaneously determine Eplerenone and its hydrolyzed metabolite in human urine. Tandem mass spectrometric detection was selected to

provide the sensitivity and selectivity needed for the 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/concentration^2) and full standard curves for human urine samples. The LLOQ is 50 ng/ml for both analytes. Acceptable precision and accuracy were obtained for concentrations above the sensitivity limit and within the standard curve range of 50–10 000 ng/ml. The upper concentration limit can be extended, with acceptable precision and accuracy, to 50 μg/ml by a 10-fold dilution with human urine blank. The extracted samples of Eplerenone and its hydrolyzed metabolite were stable at room temperature for at least 24 h. These samples can be reinjected for repeat analyses within 24 h if necessary. The validated SPE–LC-MS/MS human urine assay was specific for Eplerenone and its hydrolyzed metabolite in human urine. Endogenous compounds and other metabolites of Eplerenone in human urine did not interfere with the assay. The assay is simple, rapid and rugged with a throughput of 100 samples per run in 10 h and 200 samples on two analytical runs per day. The method was successfully applied in the determination of Eplerenone and its hydrolyzed metabolite in human urine samples for clinical studies.

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