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A single, selective and simple validated method for simultaneous estimation of amiloride and hydrochlorothiazide in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT: A single, simple and selective method for simultaneous estimation of amiloride and hydrochlorothiazide in human plasma was validated using triamterine and hydrochlorothiazide^{13}C,d2 as internal standard. The compounds were separated on a reverse-phase column with an isocratic mobile phase consisting of 2 mM ammonium acetate pH 3.0 and acetonitrile (30:70, v/v) and detected by tandem mass spectrometry with positive/negative ion mode. The analytes and internal standards were extracted from plasma using simple solid phase extraction. The ion transitions recorded in multiple reaction monitoring mode were *m/z* 230.1 → 116.0 for amiloride, *m/z* 254.1 → 237.1 for internal standard, triamterine in positive mode and *m/z* 296.1 → 204.9 for hydrochlorothiazide, *m/z* 299.2 → 205.8 for internal standard, hydrochlorothiazide^{13}C,d2 in negative ion mode. Linearity in plasma was observed over the concentration range 0.1–10 ng/mL for amiloride and 5.0–500.0 ng/mL for hydrochlorothiazide. The mean recovery was 41.1 and 81.5% for amiloride and hydrochlorothiazide respectively. The coefficient of variation of the assay was less than 11.2 and 5.2% for amiloride and hydrochlorothiazide, respectively, and the accuracy was 89.0–98.1 and 96.6–102.9% for amiloride and hydrochlorothiazide, respectively. The validated method can be applied to the pharmacokinetic study of amiloride and hydrochlorothiazide. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: amiloride; hydrochlorothiazide; triamterine; hydrochlorothiazide^{13}C,d2; LC-MS-MS; human plasma

Introduction

Amiloride (Fig. 1a; chemically 3,5-diamino-*N*-carbamimidoyl-6-chloropyrazine-2-carboxamide) is an orally administered potassium–sparing diuretic agent, widely used therapeutically, mainly in a combination formulation with hydrochlorothiazide (Fig. 1b; chemically 6-chloro-3,4dihydro-2 h-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide; British Pharmacopoeia Commission, 2003). Large-scale clinical trials demonstrated that the combination of amiloride and hydrochlorothiazide achieved the best results in terms of reduced cardiovascular morbidity and mortality (Dahlöf *et al.*, 1991). The combination has the British-approved name of co-amilozide. The joint use of amiloride and hydrochlorothiazide for the treatment of nephrogenic diabetes insipidus (Kirchlechner *et al.*, 1999), as well as of oxalate stone formation in patients with an inherited cellular defect in oxalate transport, has been proposed (Baggio *et al.*, 1986). It was also observed that administration of co-amilozide in nitroglycerin therapy has important antianginal effects, improving the exercise capacity of patients with stable angina (Parker *et al.*, 1996; Sussex *et al.*, 1994).

As amiloride and hydrochlorothiazide are widely used in combination, different methods have been reported for the estimation of amiloride and hydrochlorothiazide in pharmaceutical preparations, including polarography (Martin *et al.*, 1999), high-performance liquid chromatography (Agatonovic-kustrin *et al.*, 1998; Croo *et al.*, 1985) and spectrophotometry (Prasad *et al.*, 1998, Murat and Nevin, 1999; Mónica *et al.*, 2004). As amiloride dose is low (5 mg), a sensitive method is required for complete pharmacokinetic profiling of amiloride for bioequivalence studies. Reported methods for determination of amiloride in biological fluids include HPLC with ultraviolet (Jankowski *et al.*, 1997, Lee and Tannock, 1996; Xu *et al.*, 1991) fluorescence detection (Abdel-Hay *et al.*, 1992) and capillary isotachophoresis (Sádecká and Polonský, 1996). There are very few methods reported for simultaneous estimation of amiloride and hydrochlorothiazide. an LC-MS/MS method for simultaneous determination of amiloride and hydrochlorothiazide in human urine has been reported with a lower limit of quantification (100 ng/mL) for the analytes, which was not sufficient for estimation of amiloride in human plasma (Deventer *et al.*, 2002).

A sensitive method with LC–MS/MS using electr spray ionization involving protein precipitation as extraction technique

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Abbreviations used: MRM, multiple reaction monitoring.

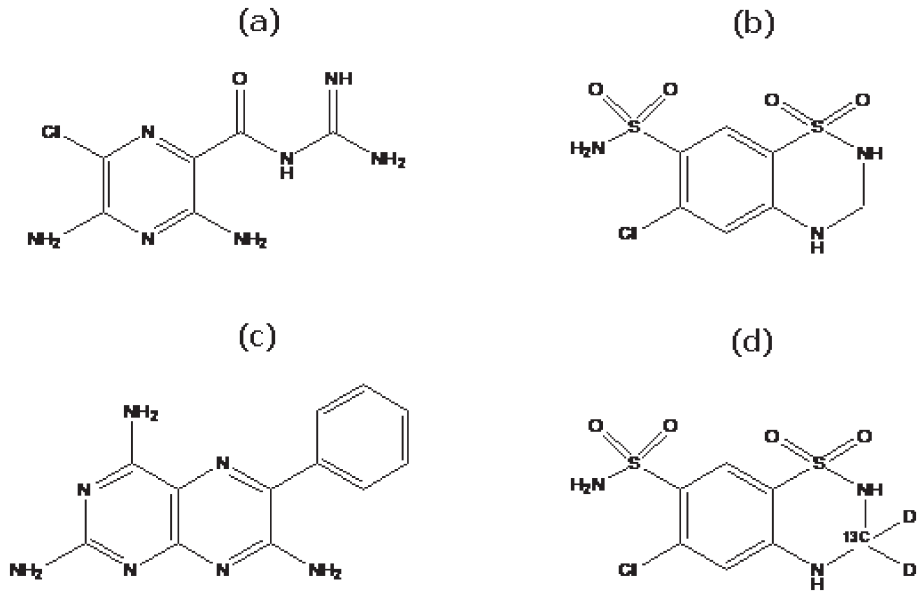

Figure 1. Chemical structure of (a) amiloride, (b) hydrochlorothiazide, (c) triamterine and (d) hydrochlorothiazide^{13}C,d2.

is reported. The chromatographic run-time was 6.5 min with a 20 µL aliquot of plasma extract injected into the column. The lower limits of quantifications were 0.1 and 1.0 ng/mL for amiloride and hydrochlorothiazide respectively (Song *et al.*, 2007).

This paper presents a simple, reliable, rapid method and validated method for simultaneous estimation of amiloride and hydrochlorothiazide in human plasma, with the advantage of a simple solid-phase extraction method which allows for a high throughput of over 160 samples per day. Solid-phase extraction was applied, which ensured much more sample cleanup, resulting in better selectivity and higher recovery. This method has a run time of 3.5 min and a 5 µL injection volume. The internal standards used were triamterine (Fig. 1c) and hydrochlorothiazide^{13}C,d2 (Fig. 1d).

Experimental

Materials and chemicals

Reference standard of hydrochlorothiazide and triamterine was obtained from IPCA Laboratories Ltd (India) and reference standards of amioloride and hydrochlorothiazide^{13}C,d2 were procured from Toronto Research Chemicals Inc. These standards had purity ≥ 98%. HPLC-grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Formic acid and ammonia, Supra grade, were procured from Merck Ltd (Mumbai, India). Ammonium acetate of Ultra grade was procured from Merck Ltd (Mumbai, India). Lichrosep DVB-HL (30 mg, 1 mL) SPE cartridges were procured from Merck Ltd (Mumbai, India). Water used in the entire analysis was prepared through a Milli-Q water purification system from Millipore (Bangalore, India). Blank human blood was collected with Na heparin as anticoagulant from healthy and drug free volunteers. Plasma was separated by centrifugation at 2061 ***g*** at 10°C, and stored at −20°C.

Liquid chromatography and mass spectrometric conditions

The liquid chromatography system (Shimadzu, Kyoto, Japan) was coupled with a mass spectrometer, API-4000 (ABS Sciex, Canada). The analytical column, Hypurity Advance (100 × 4.6 mm, 5 µm particle size) from Thermo Electron Corporation (Cheshire, UK) was used for separation of analyte and internal standard. Mobile phase of 2 mM ammonium acetate pH 3.00 ± 0.05 with acetonitrile in the ratio of 30:70 (v/v) was pumped isocratically at a flow rate of 0.5 mL/min. Autosampler temperature was set at 10°C and the injection volume was 5 µL. The column oven temperature was maintained at 40°C and the total LC run time was 3.5 min.

The MS/MS system was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion *m/z* 230.1 to the product ion *m/z* 116.0 for amiloride and the transition of the protonated molecular ion *m/z* 254.0 to the product ion *m/z* 237.1 for the internal standard, triamterine, as well as the transition of deprotonated molecular ion *m/z* 296.1 to the product ion *m/z* 204.9 for hydrochlorothiazide and the transition of the deprotonated molecular ion *m/z* 299.2 to the product ion *m/z* 205.8 for the internal standard, hydrochlorothiazide^{13}C,d2. The instrument response was optimized for amiloride, hydrochlorothiazide and internal standards by infusing a constant flow of a solution of the drug dissolved in mobile phase.

Electrospray ionization (ESI) was performed in the positive ion/negative ion mode. The source temperature temperature was 550°C. Ion spray voltages of 2200 and −4000 V were applied in positive and negative mode, respectively. Nitrogen was used as the collision gas. The curtain gas was kept at 20. The optimized GAS1 and GAS2 were 35 and 65, respectively. Compound dependant parameters set for amiloride and IS were decluster potential, 53 and 60 V; entrance potential, 10 V for both; collision energy, 45 and 37 eV; and cell exit potential, 10 and 6 V, respectively. The compound dependant parameters set for hydrochlorothiazide and IS were decluster potential, −86 and −79 V; entrance potential, −10 V for both; collision energy, −33 eV for both; and cell exit potential, −10 V for both.

Q1 and Q3 were maintained at unit resolution and the dwell time was kept at 200 ms. Nitrogen gas was used as the CAD gas. The instrument was interfaced with a computer running Analyst version 1.4.2 software.

Preparation of standards and quality control samples

A 1 mg/mL stock solution for amiloride and hydrochlorothiazide were prepared by dissolving their accurately weighted compounds in methanol. A 100 µg/mL stock was prepared for triameterine by dissolving its accurately weighed compound in 0.2% (v/v) ammonia in methanol.

A 500 µg/mL stock was prepared for hydrochlorothiazide^{13}C,d2 by dissolving its accurately weighed compound in methanol. The 1 mg/mL stock solution of amiloride and hydrochlorothiazide was serially diluted to prepare working solutions in the required concentration range with diluent methanol–water (60:40, v/v). Two separate stock solutions of amiloride and hydrochlorothiazide were prepared for bulk spiking of the calibration curve and quality control samples for the method validation experiment as well as the subject sample analysis. The calibration standards and quality control (QC) samples were prepared by spiking (5% of the total plasma volume) with working solutions. Calibration standards were prepared at concentration of 0.10, 0.20, 0.50, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00 ng/mL for amiloride and at concentration 5.00, 10.00, 25.00, 50.00, 100.00, 200.00, 300.00, 400.00 and 500.00 ng/mL for hydrochlorothiazide. Similarly, quality control standards were prepared at four different concentrations namely, 0.10 (lower limit of quantitation, LLOQ), 0.28 (low quality control, LQC), 3.50 (medium quality control, MQC) and 7.70 (high quality control, HQC) ng/mL for amiloride and at concentrations 5.00 (LLOQ), 14.00 (LQC), 175.00 (MQC) and 350.00 (HQC) ng/mL for hydrochlorothiazide. Sufficient calibration standards and quality control standards were prepared to validate the method and to serve as standards and controls during the assay of all study samples. However during the study, only three levels of controls were prepared as LQC, MQC and HQC. Aliquots of the standards and quality controls were stored together with the study samples at −70°C until used for sample processing.

Extraction procedure

The plasma samples (300 µL) were transferred to 1.7 mL clear tubes (Tarsons, India) to which were added 20 µL of internal standard (working solution of 0.050 µg/mL of triameterene and 6.000 µg/mL of hydrochlorothiazide ^{13}C,d2). The samples were vortexed to mix. A 300 µL aliquot of 0.01% (v/v) ammonia in water was added to each sample. The samples were vortexed for 30 s and centrifuged for 5 min at 14,000 rpm. After centrifugation the samples were loaded on Lichrosep DVB-HL 30 mg/1 mL cartridges pre-conditioned with 1 mL methanol followed by 1 mL of 0.01% (v/v) ammonia in water. The plasma matrix was drained out from the extraction cartridges by applying positive nitrogen pressure. The extraction cartridges washed with1 mL of 0.01% (v/v) ammonia in water followed by 1 mL of 10% (v/v) methanol in water. The analyte and the internal standard were eluted with 1 mL of elution solution and 0.1% formic acid in acetonitrile (v/v). The eluent were dried under nitrogen gas at 45°C. After drying the sample were reconstituted with 400 µL reconstitution solution: 2 mM ammonium acetate in water (pH 3.00 ± 0.05)–acetontrile (10:90). A 5 µL aliquot of the sample was injected into the LC-MS-MS system through the autosampler.

Method validation

Selectivity. Selectivity was performed using 10 different sources of blank plasma comprising six normal, two hemolyzed and two lipemic. They were processed as per the extraction method and their responses were assessed at the retention time of analytes. The internal standards with six LLOQ samples for amiloride and hydrochlorothiazide were prepared from the screened blank plasma samples which had the least interference.

Carry over. Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port was able to avoid any carry-forward of injected sample in subsequent runs. The design of the experiment comprised blank plasma, LLOQ and upper limit of quantitation (ULOQ) followed by blank plasma to check for any possible interference due to carryover.

Linearity and lower limit of quantification. The linearity of the method was determined by analysis of five standard plots associated with a nine-point standard calibration curve. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually using least square weighted ($1/x^2$) linear regression. The calculation was based on the peak area ratio of analyte vs the area of internal standard. The concentration of the analyte was calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with the reciprocate of the drug concentration as a weighing factor ($1/x^2$). Several regression types were tested and the linear regression (weighted with 1/concentration^2) was found to be the simplest regression, giving the best results ($r^2 \geq 0.9986$) for amiloride and ($r^2 \geq 0.9994$) for hydrochlorothiazide. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least five times more than that of drug-free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0% and for for LLOQ it should not be more than ±20.0%.

Accuracy and precision. The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the four quality control levels on three different days. In each of the precision and accuracy batches, six replicates at each quality control level were analyzed. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of relative error (%RE) and coefficient of variation (%CV), respectively.

Matrix effect. The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal controlled plasma samples in replicate ($n = 4$). LQC and HQC working solutions were spiked post-extraction in duplicate for each lot. The results found were well within the acceptable limit set, i.e. the RSD of area ratio was within ±15% at each level tested. Also, the ion suppression/enhancement of analyte signal due to endogenous matrix interferences does not affect the quantification of analyte and IS peak, which was confirmed by post-column infusion experiment. A standard solution containing amiloride and hydrochloride (at MQC level) and IS was infused post-column via a 'T' connector into the mobile phase at 10 µL/min, employing an in-built infusion pump. Aliquots of 5 µL of extracted control plasma were then injected into the column by the autosampler and acquired. Any dip in the baseline of respective MRM upon injection of double blank plasma (without IS) would indicate ion suppression, while a peak at the retention time of analyte in respective would indicate ion enhancement.

Recovery. Absolute recoveries of the analyte were determined at the three different quality control levels viz. LQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the unextracted standard mixtures (prepared in the elution solution at the same concentrations as the extracted samples) representing 100% recovery.

Dilution integrity. The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of half and quarter concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4, respectively, against the freshly prepared calibration curve.

Stability. All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against comparison samples of identical concentration. Stock solutions of analyte and IS were checked for short-term stability at room temperature and long-term stability at 2–8°C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Bench-top stability, autosampler stability (process stability), freeze–thaw stability and long-term stability in plasma were performed at LQC and HQC level using six replicates at each level. Freeze–thaw stability was evaluated by successive cycles of freezing (at −20°C) and thawing (without warming) at room temperature. To meet the acceptance criteria, the difference between the stability and fresh samples should be within ±15%.

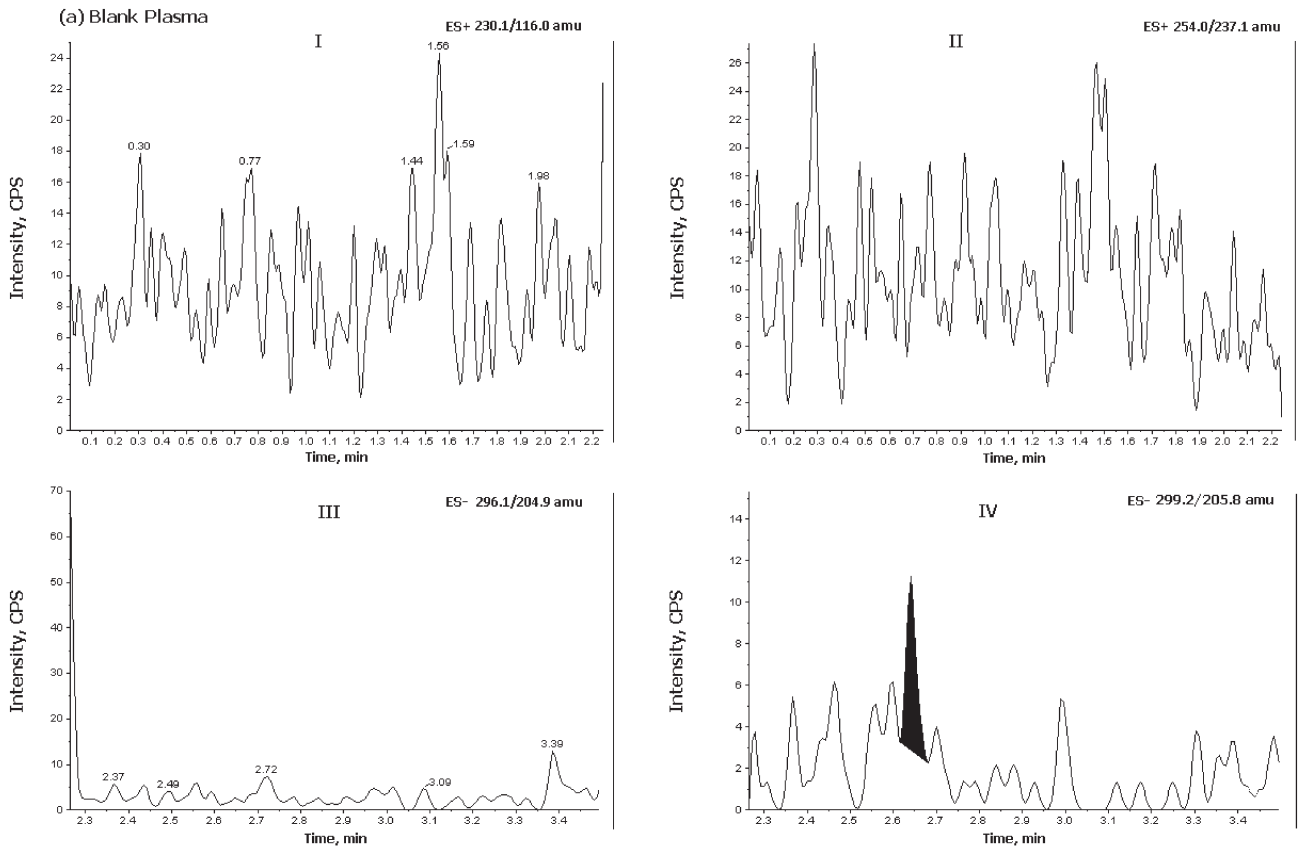

Figure 2. Chromatogram of amiloride (I), triamterine (internal standard, II), hydrochlorothiazide (III) and hydrochlorothiazide^{13}C,d2: (a) blank plasma; (b) blank plasma spiked at LLOQ level (CS-1, 0.1 and 5 ng/mL).

Results and discussion

The mean absolute recoveries of amiloride determined at 0.28, 3.50 and 7.70 ng/mL were 39.8 (RSD 8.7%), 42.31 (RSD 2.6%) and 41.2% (RSD 3.1%), respectively. The mean absolute recoveries of hydrochlorothiazide determined at 5.00, 14.00 and 175.0 ng/mL were 86.4% (RSD 3.7%), 77.1% (RSD 9.0%) and 81.5% (RSD 5.7%), respectively. The mean absolute recovery of triameterine and hydrochlorothiazide^{13}C,d2 were 40.0% (RSD 4.3%) and 88.3% (RSD 3.1%) respectively.

Minimal matrix effect for amiloride and hydrochlorothiazide was observed from the six different plasma lots tested. The RSDs of the area ratios of post-spiked recovery samples at LQC and HQC levels were less than 5.1% for amiloride and 2.0% for hydrochlorothiazide. For the internal standard the RSDs of the area ratios over both LQC and HQC levels were less than 7.3 and 5.6% for triameterine and hydrochlorothiazide^{13}C,d2 respectively. This indicated that the extracts were 'clean' with no co-eluting compounds influencing the ionization of the analyte and the internal standard.

The high selectivity of MS-MS detection allowed the development of a very specific and rapid method for the determination of amiloride and hydrochlorothiazide in plasma. Representative chromatograms obtained from blank plasma and blank plasma spiked with LLOQ standard are presented in Fig. 2(a and b), respectively. No significant interfering peak of endogenous compounds was observed at the retention time of analyte in blank human plasma containing Na heparin as the anti-coagulant in 10n different plasma lots which was compared vs six replicates of extracted samples at the LLOQ level.

The LLOQ, defined as that concentration of analyte that can still be determined with acceptable precision (%RSD < 20) and accuracy (bias within ±20%) was found to be 0.1 ng/mL for amiloride and 5 ng/mL for hydrochlorothiazide. Results of the intra-batch and inter-batch validation assays for amiloride are presented in Tables 1 and 2, respectively. The inter- and intra-batch precisions were ≤ 9.0 and ≤ 11.2%, whereas the inter- and intra-batch accuracies in terms of %bias were −11.0 to −1.9 and −10.0 to 5.7, respectively, for amiloride. Results of the intra- and inter-batch validation assays for hydrochlorothiazide are presented in Tables 1 and 2, respectively. The inter- and intra-batch precisions were ≤ 5.2 and ≤ 9.2%, whereas the inter- and intra-batch accuracies in terms of %bias were −3.4–2.9 and −2.9–7.2, respectively, for hydrochlorothiazide.

Bench-top and processed (autosampler) stabilities for amiloride and hydrochlorothiazide were performed at LQC and HQC levels. The results revealed that amiloride and hydrochlorothiazide were stable in plasma for at least 7 h at room temperature and 54 h in the autosampler at 10°C. It was confirmed that repeated freeze and thawing (five cycles) of spiked plasma samples at LQC and HQC level did not affect the stability of amiloride and hydrochlorothiazide. Amiloride and hydrochlorothiazide were found to be stable for a minimum of five freeze and thaw cycles. The long-term stability results also indicated that amiloride were stable in human plasma for up to 56 days at a storage temperature of −70°C.

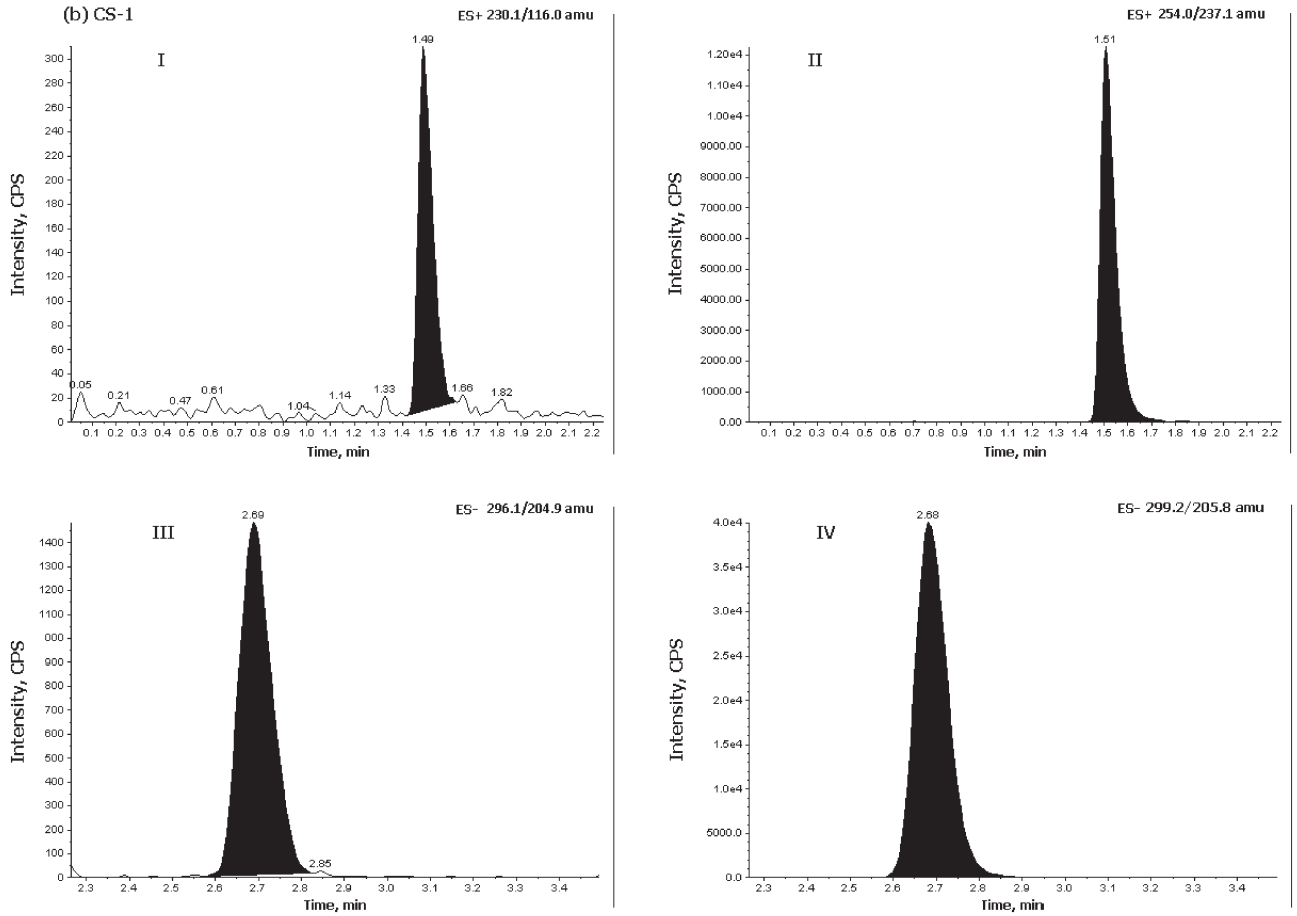

Figure 2. *Continued.*

Table 1. Intra-batch precision and accuracy ($n = 6$) of amiloride and hydrochlorothiazide in human plasma

Analyte	Spiked concentration (ng/mL)	Mean calculated concentration (ng/mL)	%RSD	%Bias
Amiloride	0.100	0.090	8.9	−10.0
	0.280	0.278	2.2	−0.7
	3.500	3.700	3.6	5.7
	7.700	7.461	9.0	−3.1
Hydrochlorothiazide	5.000	5.362	3.1	7.2
	14.000	14.559	1.0	4.0
	175.000	179.330	1.6	2.5
	385.000	373.990	9.2	−2.9

Table 2. Inter-batch precision and accuracy ($n = 6$) of amiloride and hydrochlorothiazide in human plasma

Analyte	Spiked concentration (ng/mL)	Mean calculated concentration (ng/mL)	%RSD	%Bias
Amiloride	0.100	0.089	11.2	−11.0
	0.280	0.264	4.6	−5.7
	3.500	3.432	6.8	−1.9
	7.700	7.397	6.6	−3.9
Hydrochlorothiazide	5.000	5.147	3.7	2.9
	14.000	13.983	3.1	−0.1
	175.000	169.809	4.4	−3.0
	385.000	371.822	5.2	−3.4

During method development different options were evaluated to optimize sample extraction, detection parameters and chromatography. In the nonionic forms, the strong binding of analytes to the copolymer of SPE cartridge enables sufficient cleanup. The best signal for the amiloride was achieved with the ESI positive ion mode and for hydrochlorothiazide with the ESI negative ion mode. A mobile phase containing buffer ammonium acetate and formate salt at different molarity and acetonitrile in varying combinations was tried during the initial development stages. The effect of pH of the buffer on sensitivity and peak shape was also checked. However, the best signal and peak shape for amiloride and hydrochlorothiazide were achieved using a mobile phase of 2 mM ammonium acetate pH 3.0 ± 0.05 in deionized water in combination with acetonitrile (30:70 v/v). Use of a Hypurity Advance (100 × 4.6 mm, 5 µm) column resulted in better separation, good peak shape and reduced run time. The retention times for amiloride and triamterene were ~1.49 and ~1.52 min, respectively. The retention times for hydrochlorothiazide and Hydrochlorothiazide^{13}C,d2 were ~2.69 and ~1.68 min, respectively. The chromatographic separation between amiloride and hydrochlorothiazide along with respective internal standard was sufficient for polarity switching.

Triamterine used as internal standard belonged to the same therapeutic category as amiloride. Ionization, retention and extraction characteristics were found to be similar to that of amiloride and hence it was selected as the internal standard of choice. Labeled internal standard, hydrochlorothiazide^{13}C,d2, was used as the internal standard for hydrochlorothiazide. The validated method can be employed for pharmacokinetic studies for simultaneous estimation of amiloride and hydrochlorothiazide in human plasma.

Conclusion

A simple, selective and rapid method for the simultaneous estimation of amiloride and hydrochlorothiazide in human plasma was developed and validated, using high-performance liquid chromatographic separation and electronspray ionization tandem mass spectrometric detection in positive/negative mode. The validated method can be applied to pharmacokinetic studies for simultaneous estimation of amiloride and hydrochlorothiazide. This method is an excellent analytical option for rapid and simultaneous quantification of amiloride and hydrochlorothiazide in human plasma.

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