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ORIGINAL RESEARCH

Sensitive liquid chromatography–tandem mass spectrometry method for quantification of hydrochlorothiazide in human plasma

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ABSTRACT: A simple, rapid, sensitive and specific liquid chromatography–tandem mass spectrometry method was developed and validated for quantification of hydrochlorothiazide (I), a common diuretic and anti-hypertensive agent. The analyte and internal standard, tamsulosin (II) were extracted by liquid–liquid extraction with diethyl ether–dichloromethane (70:30, v/v) using a Glas-Col Multi-Pulse Vortexer. The chromatographic separation was performed on a reversed-phase column (Waters symmetry C_{18}) with a mobile phase of 10 mM ammonium acetate–methanol (15:85, v/v). The protonated analyte was quantitated in negative ionization by multiple reaction monitoring with a mass spectrometer. The mass transitions *m/z* 296.1 → 205.0 and *m/z* 407.2 → 184.9 were used to measure I and II, respectively. The assay exhibited a linear dynamic range of 0.5–200 ng/mL for hydrochlorothiazide in human plasma. The lower limit of quantitation was 500 pg/mL, with a relative standard deviation of less than 9%. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. A run time of 2.5 min for each sample made it possible to analyze a throughput of more than 400 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: hydrochlorothiazide; LC-MS/MS; human plasma; liquid–liquid extraction; pharmacokinetic study

INTRODUCTION

Hydrochlorothiazide, see Fig. 1, and the other thiazide diuretics have been used in antihypertensive therapy since the advent of chlorothiazide in 1957, often in combination with other antihypertensive drugs such as beta blockers, angiotensin-converting enzyme (ACE) inhibitors, or more recently, angiotensin II receptor blockers (ARBs) (Wellington and Faulds, 2002).

Several liquid chromatographic methods have been reported for determining hydrochlorothiazide concentration in plasma (Niopas and Daftsios, 2002; Miller and Amestoy, 1992; Medvedovici *et al.*, 2000; Richter *et al.*, 1996; de Vries and Voss, 1993; Kuo *et al.*, 1990; Azumaya, 1990; Takubo *et al.*, 2004; Zendelovska *et al.*, 2004). All of these methods employed liquid–liquid extraction except for Zendelovska *et al.* (2004) who employed solid-phase extraction on RP-select B cartridges followed by isocratic reversed-phase chromatography on a Hibar Lichrospher 100 RP_8 column with UV detection at 230 nm. The limit of quantification was

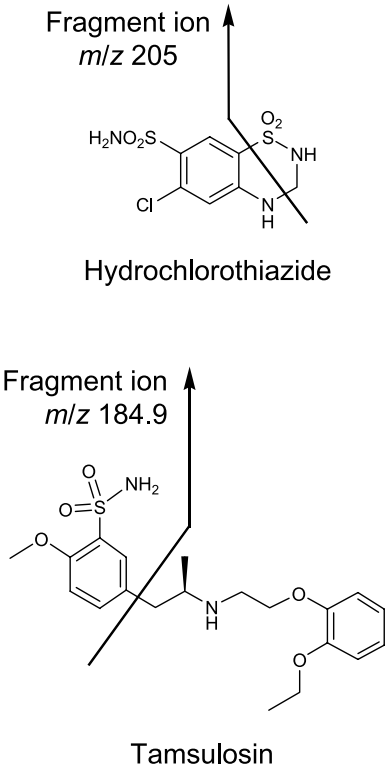

Figure 1. Chemical structures and proposed fragmentation pathway for hydrochlorothiazide and IS (tamsulosin).

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Abbreviations used: ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

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10 ng/mL for a 1 mL plasma sample. The liquid–liquid extraction methods involved several steps yielding poor separation from the plasma endogeneous interferences and gave highly variable and relatively low recoveries.

Kuo *et al.* (1990) reported a column-switching high-performance liquid chromatographic method with ultraviolet detection (LC-UV) for determining hydrochlorothiazide in plasma from 25 to 2000 ng/mL. Niopas and Daftsios (2002) determined hydrochlorothiazide in human plasma in the range from 5 to 80 ng/mL by the LC-UV method using a 1 mL sample. Takubo *et al.* (2004) reported a liquid chromatography–electrospray ionization tandem mass spectrometry method (LC-MS-MS) for the determination of hydrochlorothiazide in rat plasma over the range 4–1000 ng/mL. The post-column addition technique was adopted to enhance the negative ionization of hydrochlorothiazide and raise the sensitivity for quantification of hydrochlorothiazide. In all reported methods, plasma volume requirement was high, chromatographic run time was longer and sensitivity not adequate for pharmacokinetic studies.

It is well known that HPLC tandem MS (MS-MS) further enhances specificity and provides an improved signal-to-noise ratio compared with single-stage MS (Willoughby *et al.*, 2002). The purpose of this work was to explore the high selectivity and sensitivity of triple quadrupole MS system operated in MS-MS mode with an ESI interface for the development and validation of a robust reversed-phase LC-MS-MS method for hydrochlorothiazide quantification in human plasma. It was essential to establish an assay capable of quantifying hydrochlorothiazide at concentrations down to 500 pg/mL. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of hydrochlorothiazide.

In recent years, a number of laboratories have reported the use of high-throughput bioanalytical procedures using LC-MS-MS (Jemal, 2000; Ramakrishna *et al.*, 2004a–e, 2005a–c). Our method is simple, rapid, robust, specific and sensitive that makes it an attractive procedure in high-throughput bioanalysis.

EXPERIMENTAL

Chemicals. Hydrochlorothiazide reference standard (99.3% pure) was obtained from Torrent Pharmaceuticals Limited (Ahmedabad, India). Tamsulosin was employed as an internal standard (IS) and was obtained from our R&D department. Chemical structures are presented in Fig. 1. Drug-free human plasma, containing EDTA as an anticoagulant, was obtained from the Usha Mullapudi Cardiac Center (Hyderabad, India). Stock solutions of hydrochlorothiazide (1 mg/mL) and IS (1 mg/mL) were separately prepared in 10 mL volumetric flasks with methanol. HPLC-grade LiChrosolv methanol and LiChrosolv acetonitrile were from Merck (Darmstadt, Germany). Diethyl ether and dichloromethane were from Merck (Worli, Mumbai, India). HPLC type I water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

LC-MS/MS instrument and conditions. The HPLC, Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) was equipped with a G1312A binary pump, a G1379A degasser, a G1367A autosampler equipped with a G1330B thermostat, a G1316A thermostatted column compartment and a G1323B control module. The chromatography was on a Waters Symmetry®, C_{18} column (5.0 µm, 150 × 4.6 mm i.d.) at 30°C. The mobile phase composition was a mixture of 10 mM ammonium acetate–methanol (15:85, v/v), which was pumped at a flow-rate of 1.0 mL/min.

Mass spectrometric detection was performed on an API 3000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A turbo ionspray interface in negative ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on Analyst 1.4 software package (SCIEX).

Sample processing. A 500-µL volume of plasma sample was transferred to a 15-mL glass test tube, and then 25 µL of IS working solution (1.0 µg/mL) was spiked. After vortexing for 30 s, a 4 mL aliquot of extraction solvent, diethyl ether–dichloromethane (70/30) was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The sample was vortex-mixed for 3 min using Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The organic layer (3 mL) was transferred to a 5 mL glass tube and evaporated to dryness using TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 200 µL of diluent (water–methanol, 50:50, v/v) and a 20 µL aliquot was injected into the chromatographic system.

Bioanalytical method validation. Working solutions for calibration and controls were prepared from the stock solution by dilution using water–methanol (1:1). The IS working solution (1 µg/mL) was prepared by diluting its stock solution with water–methanol (1:1). Working solutions (25 µL) were added to 475 µL drug-free plasma to obtain hydrochlorothiazide concentration levels of 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL. Quality control (QC) samples were prepared as a bulk, at concentrations of 0.5 (LLOQ), 1.5 (low), 100 (medium) and 160 ng/mL (high).

A calibration curve was constructed from a blank sample (a plasma sample processed without an IS), a zero sample (a plasma processed with IS) and nine non-zero samples covering the total range (0.5–200 ng/mL), including lower limit of quantification (LLOQ). Such calibration curves were generated on five consecutive days. Linearity was assessed by a weighted ($1/x$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal

Table 1. Tandem mass-spectrometer main working parameters

Parameter	Value
Source temperature, °C	200
Dwell time per transition, ms	200
Nebulizer gas, psi	9
TurboIon gas, psi	8
Curtain gas, psi	14
Collision gas, psi	5
Ion spray voltage, V	−4000
Entrance potential, V	−10
Declustering potential (DP), V	−60 (Analyte) and −60 (IS)
Collision energy, V	−22 (Analyte) and −30 (IS)
Collision cell exit potential, V	−15 (Analyte) and −15 (IS)
Mode of analysis	Negative
Ion transition for hydrochlorothiazide, *m/z*	296.1/205.0
Ion transition for tamsulosin, *m/z*	407.2/184.9

value except LLOQ, which was set at 20%. At least 67% of non-zero standards should meet the above criteria, including acceptable LLOQ and upper limit of quantitation.

The within-batch precision and accuracy were determined by analyzing six sets of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing six sets of quality control samples on three different batches. The quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the batch or (c) at the end of the batch. The acceptance criteria of within- and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of concentrations and the accuracy was $100 \pm 20\%$ or better for LLOQ and $100 \pm 15\%$ or better for the rest of concentrations.

Recovery of hydrochlorothiazide from the extraction procedure was determined by a comparison of the peak area of hydrochlorothiazide in spiked plasma samples (six low and high quality controls) to the peak area of hydrochlorothiazide in samples prepared by spiking extracted drug-free plasma samples with the same amounts of hydrochlorothiazide at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted quality control samples ($n = 10$) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

RESULTS AND DISCUSSION

In order to develop a method with the desired LLOQ (500 pg/mL), it was necessary to use MS-MS detection, as MS-MS methods provide improved limit of detection (LOD) for trace-mixture analysis (Jemal, 2000). The inherent selectivity of MS-MS detection was also expected to be beneficial in developing a selective and sensitive method. The product ion mass spectrum of hydrochlorothiazide and the IS are shown in Figs 2 and 3, respectively. $[M\text{-}H]^-$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from *m/z* 296.1 to 205.0 for hydrochlorothiazide and from *m/z* 407.2 to 184.9 for the IS. LC-MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity, selectivity and specificity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

Method development

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS-MS analyses. Four organic solvents, diethyl ether, hexane, ethyl acetate, dichloromethane and their mixtures in different combinations and ratios were evaluated. Finally, a mixture of diethyl ether and dichloromethane (7:3, v/v) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample and yield the highest recovery for the analyte from the plasma.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. It was found that a mixture of 10 mM ammonium acetate–methanol (15:85, v/v) could achieve our purpose and was finally adopted as the mobile phase for the chromatographic separation. A high proportion of organic solvent [10 mM ammonium acetate–methanol (15:85, v/v)] eluted the analyte and the IS at retention times of 1.6 and 1.9 min, respectively. A flow-rate of 1.0 mL/min

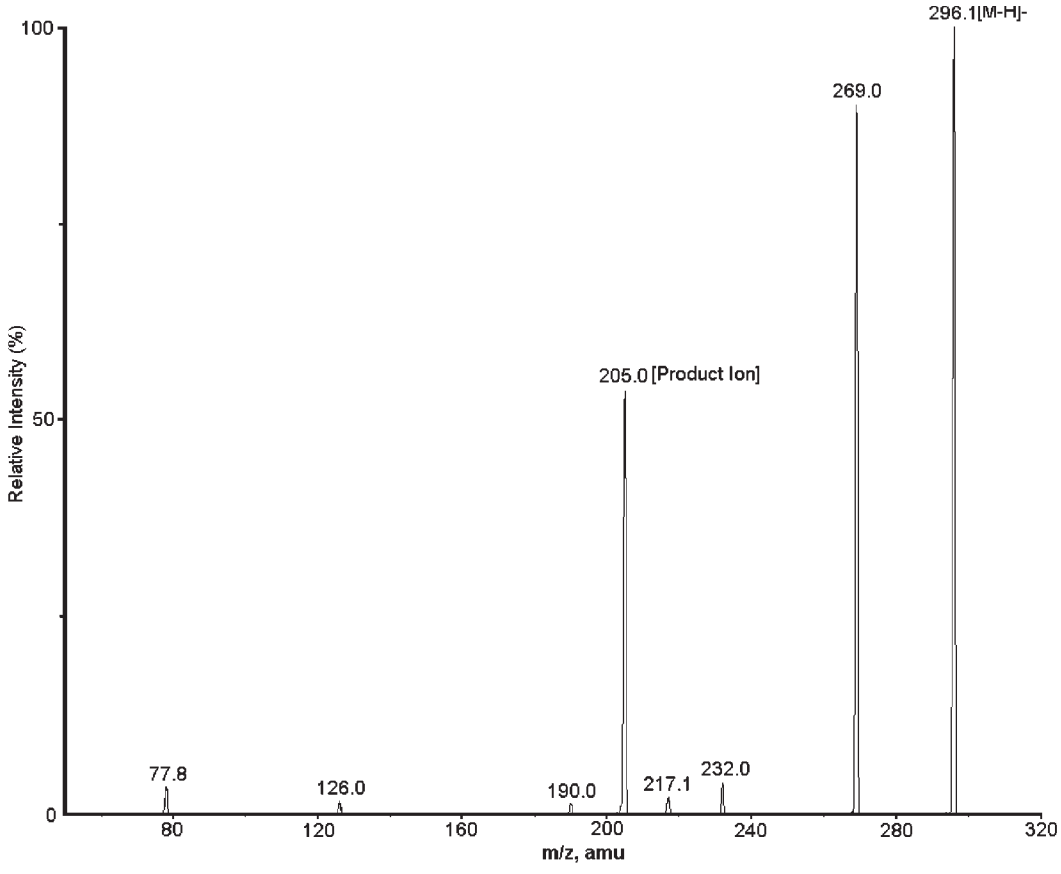

Figure 2. Full-scan negative ion turboIonspray product ion mass spectra of hydrochlorothiazide.

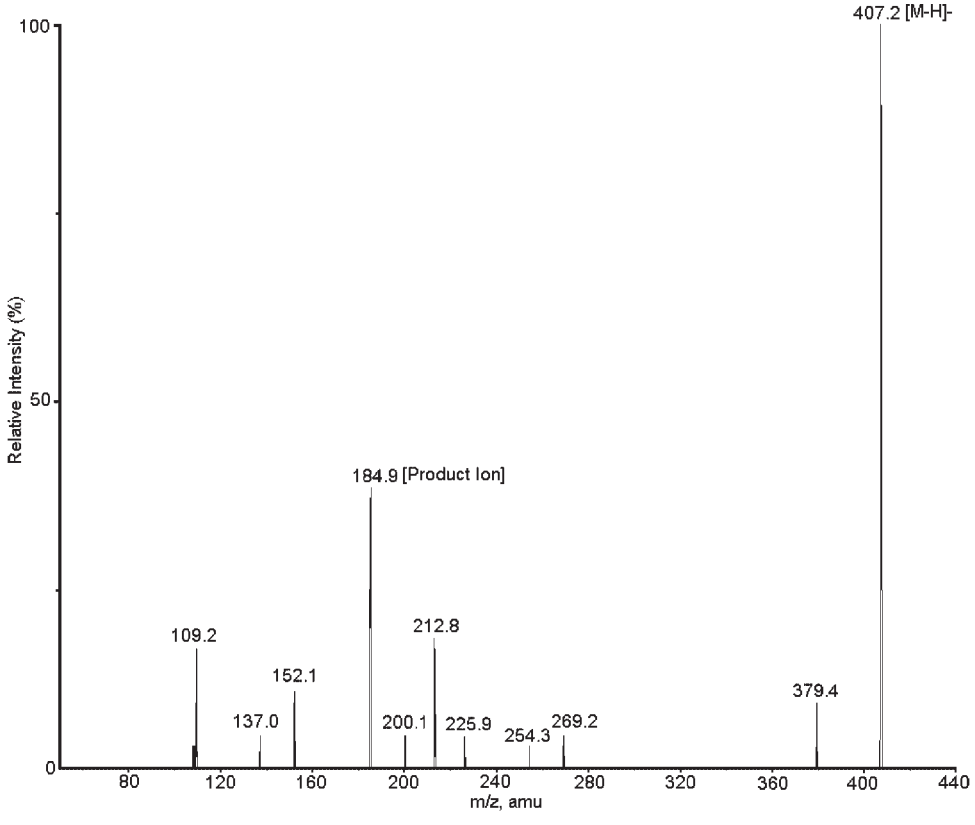

Figure 3. Full-scan negative ion turboIonspray product ion mass spectra of IS (tamsulosin).

produced a good peak shape and brought the run time to 2.5 min.

An internal standard is necessary for determination of analyte in biological samples. For an LC-MS/MS analysis, utilization of stable isotope-labeled drugs as internal standards proves helpful when significant matrix effect occurs. However, there are also many problems with the use of stable isotope-labeled internal standards. The major problems involve inadequate isotopic purity and stability, which often have an unfavorable impact on highly sensitive quantitative analyses. In the initial stages of our work, several compounds were tried to find a suitable internal standard and finally tamsulosin was found to be optimal for our work. Clean chromatograms were obtained and no significant matrix effect was found.

The calibration curve was linear over the concentration range 0.5–200 ng/mL for the analyte. The eight-point calibration curve gave acceptable results for the analyte and was used for all the calculations. The calibration model was selected based on the analysis of the data by linear regression with or without intercepts and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The residuals improved by weighted ($1/x^2$) least-squares linear regression. The best fit for the calibration curve could be achieved with the linear equation $y = mx + c$ with a $1/x^2$ weighing factor. The linear regression equation of a calibration curve for the analyte was $y = 0.0229x + 0.0015$ where y was the peak area ratio of the analyte to the IS and x was the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.999 for the analyte. Table 2 summarizes the calibration curve results for the analyte. The calibration curve obtained as described above was suitable for generation of acceptable data for the concentrations of the analyte in the samples during the validations.

Specificity

The specificity of the method was examined by analyzing blank human plasma extract (Fig. 4) and an extract spiked only with the internal standard (Fig. 5). As shown in Fig. 4, no significant interference in the blank plasma traces was seen from endogenous substances in drug-free human plasma at the retention time of the analyte. Figure 5 shows the absence of interference from the internal standard to the MRM channels of the analyte. Figure 6 depicts a representative ion-chromatogram for the lower limit of quantitation (LLOQ, 0.5 ng/mL) of the calibration curve. Excellent sensitivity was observed for 20 µL injection volume (LLOQ) corresponding to ca. 10 pg on-column. The product ion chromatogram obtained from an extracted plasma sample of a healthy subject who participated in a bioequivalence study conducted on 24 subjects is depicted in Fig. 7. Hydrochlorothiazide was unambiguously identified and was quantified as 19.3 ng/mL.

Owing to the components of the sample matrix, signal suppression or enhancement may occur. These matrix effects in the LC-MS/MS method were evaluated by spiking blank plasma extracts with low and high QC samples. The resulting chromatograms were compared with chromatograms of pure samples equally concentrated. Six independent plasma lots were used with six samples from each lot. The results (data was not shown) showed that there was no significant difference for peak responses between these samples. This effect is most likely due to the sample clean-up with LLE.

The extraction recovery of hydrochlorothiazide was 59.3% on average, and the dependence on concentration is negligible. The recovery of the IS was 63.3% at the concentration used in the assay (1 µg/mL). Recovery of the analyte and IS were low, but it was consistent, precise and reproducible. With the consistency in the recovery of hydrochlorothiazide and IS, the assay has proved to be robust in high-throughput bioanalysis.

Lowest concentration

The lower limit of quantitation (LLOQ) of hydrochlorothiazide in human plasma assay was 0.5 ng/mL.

Table 2. Precision and accuracy data of back-calculated concentrations of calibration samples for hydrochlorothiazide in human plasma

Concentration added (ng/mL)	Concentration found (mean ± SD, $n = 5$; ng/mL)	Precision (%)	Accuracy (%)
0.5	0.517 ± 0.041	7.9	103.4
1	1.026 ± 0.087	8.4	102.6
2	1.975 ± 0.068	3.4	98.8
5	5.034 ± 0.266	5.3	100.7
10	9.816 ± 0.481	4.9	98.2
20	20.436 ± 0.810	4.0	102.2
50	46.820 ± 1.658	3.5	93.6
100	97.855 ± 2.500	2.6	97.9
200	202.710 ± 2.610	1.3	101.4

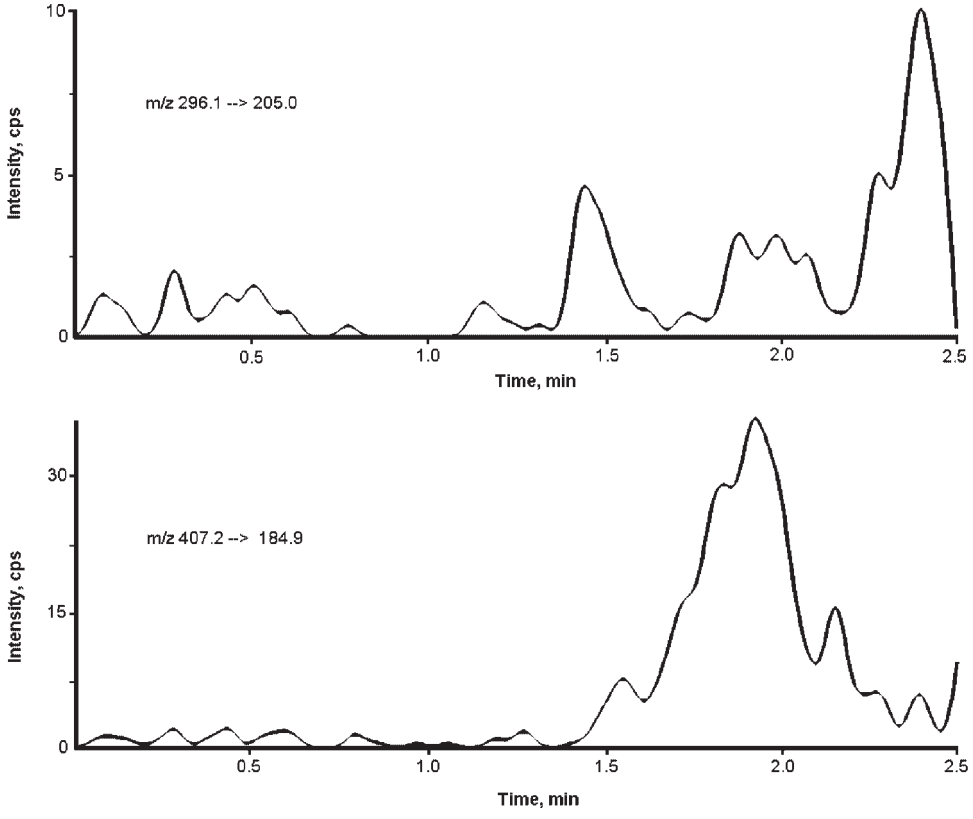

Figure 4. MRM ion-chromatograms resulting from the analysis of blank (drug and IS-free) human plasma for hydrochlorothiazide and IS.

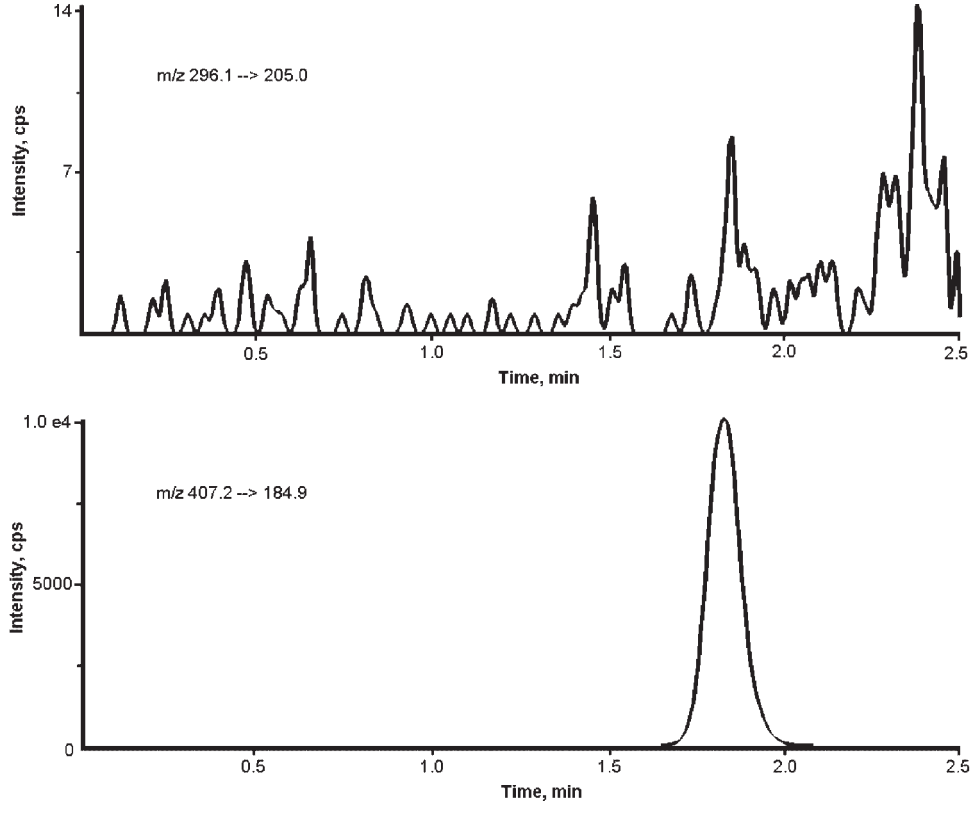

Figure 5. MRM ion-chromatograms resulting from the analysis of blank (drug-free spiked with IS) human plasma for hydrochlorothiazide and IS.

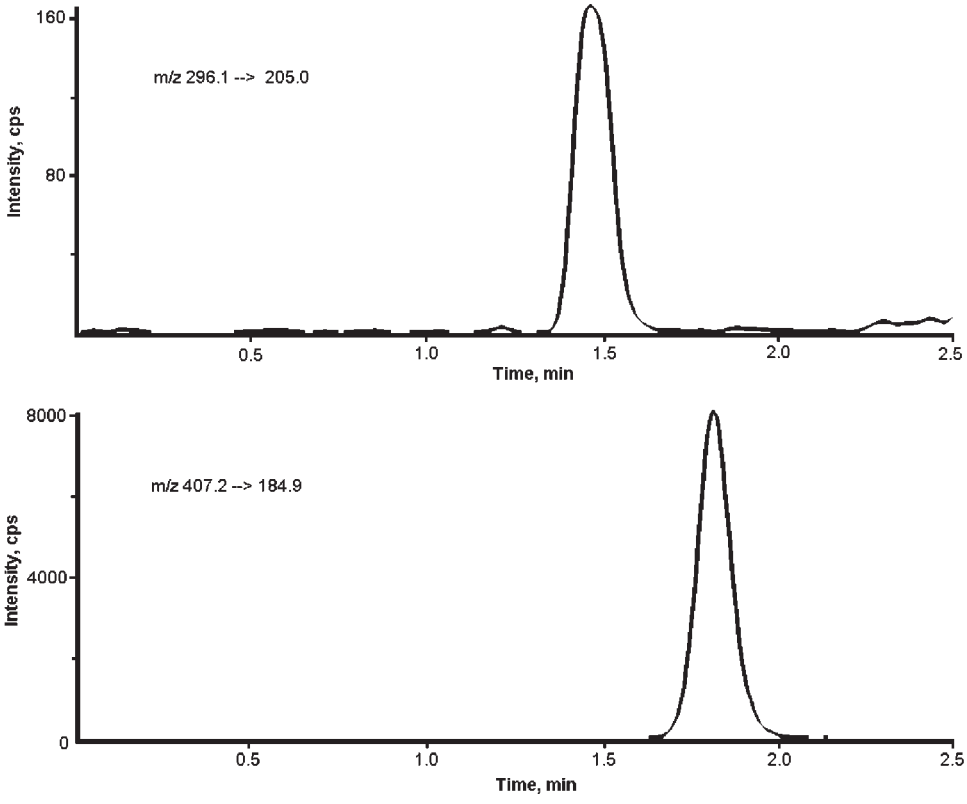

Figure 6. Representative MRM ion-chromatograms resulting from the analysis of 0.5 ng/mL (LLOQ) of hydrochlorothiazide spiked with the IS.

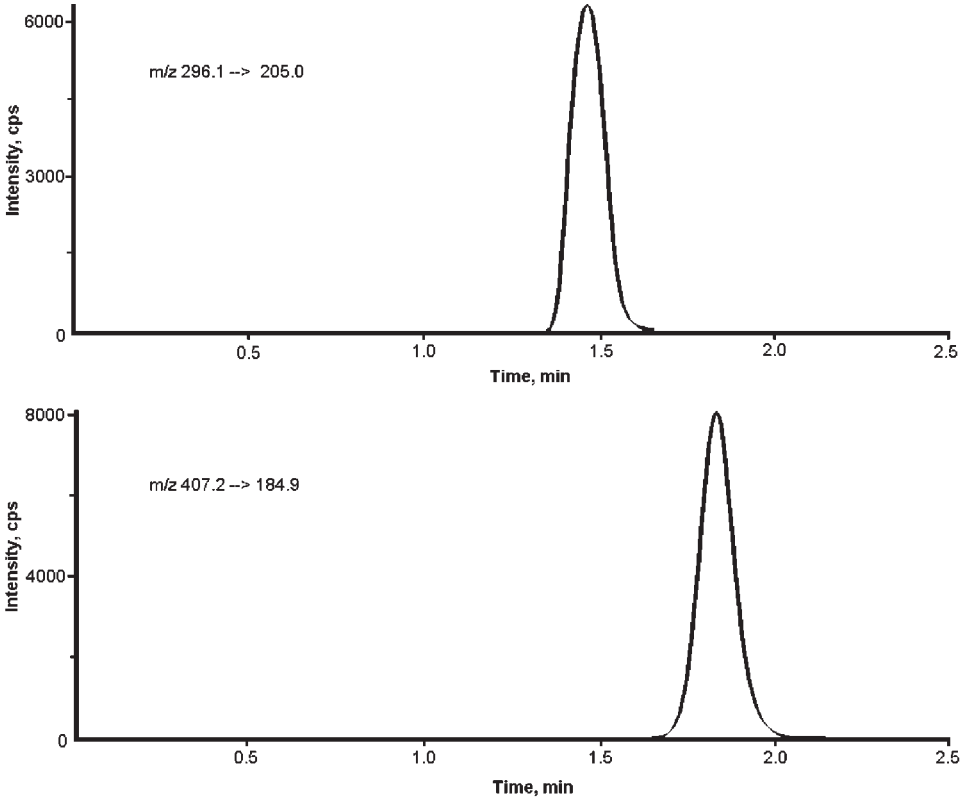

Figure 7. MRM ion-chromatograms resulting from the analysis of subject plasma sample after the administration of 12.5 mg oral single dose of hydrochlorothiazide. The sample concentration was 19.3 ng/mL.

Table 3. Precision and accuracy of the LC-MS/MS method for determining hydrochlorothiazide concentrations in plasma samples

	Within-batch precision ($n = 6$)			Between-batch precision ($n = 3$)		
Concentration added (ng/mL)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
0.5	0.512 ± 0.046	9.0	102.4	0.532 ± 0.031	5.7	106.3
1.5	1.486 ± 0.094	6.4	99.1	1.479 ± 0.061	4.1	98.6
100	97.969 ± 2.254	2.3	98.0	96.763 ± 1.358	1.4	96.8
160	149.237 ± 2.321	1.6	93.3	153.441 ± 2.969	1.9	96.5

Table 4. Stability of human plasma samples of hydrochlorothiazide

Sample Concentration (ng/mL; $n = 6$)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability for 24 h in plasma			
1.5	1.46	4.7	97.6
160	170.29	2.2	106.4
Three freeze–thaw cycles			
1.5	1.38	6.4	92.0
160	173.01	2.3	108.1
Autosampler stability for 25 h (after extracting and reconstitution)			
1.5	1.34	5.6	89.6
160	160.27	5.9	100.1
Stability for 30 days at <–50°C			
1.5	1.36	9.7	90.6
160	159.32	6.3	99.5

The between-batch precision at the LLOQ was 5.7%. The between-batch accuracy was 106.3% (Table 3). The within-batch precision was 9.0% and the accuracy was 102.4% for hydrochlorothiazide.

Middle and upper concentrations

The middle and upper quantitation levels of hydrochlorothiazide ranged from 1.5 to 160 ng/mL in human plasma. For the between-batch experiment, the precision ranged from 1.9 to 4.1% and the accuracy ranged from 96.5 to 98.6% (Table 3). For the within-batch experiment, the precision and accuracy for the analyte met the acceptance criteria (<±15%) and precision was below 7% at all concentrations tested.

Stability

The stability of the analytes in human plasma under different temperature and timing conditions, as well as the stability of the analytes in stock solution, was evaluated as follows.

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeded that expected to be encountered during the routine sample preparation (around 24 h). Samples were extracted and analyzed as mentioned above. Results are given in Table 4. Short-term stability indicated reliable stability behavior under the experimental conditions of the regular batches.

The post-preparative stability (autosampler stability) of QC samples kept in the autosampler for 25 h was also assessed. The results indicate that hydrochlorothiazide and IS can remain at the autosampler temperature for at least 25 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time (Table 4).

The data that represent the stability of hydrochlorothiazide plasma samples at two QC levels over three cycles of freeze and thaw are given in Table 4. The performed tests indicate that the analyte is stable in human plasma for three cycles of freeze and thaw, when stored at below –50°C and thawed to room temperature.

Table 4 also summarizes the long-term stability data of hydrochlorothiazide in plasma samples stored for a period of 30 days at below –50°C. The stability study of hydrochlorothiazide in human plasma showed reliable stability behavior as the means of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicated that storage of hydrochlorothiazide plasma samples at below –50°C is adequate, and no stability-related problems would be expected during the routine analysis of samples for pharmacokinetic, bioavailability or bioequivalence studies.

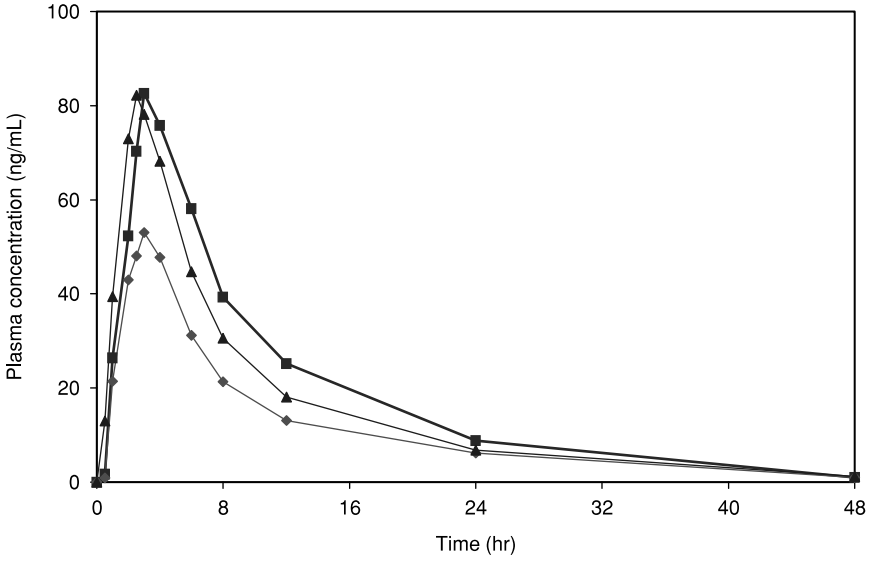

Figure 8. Representative data showing plasma concentration–time profiles of three healthy subjects after the administration of an oral single dose of 12.5 mg of hydrochlorothiazide.

The stability of stock solutions was tested and established at room temperature for 2 and 40 h and under refrigeration for 30 days. The recoveries for hydrochlorothiazide and tamsulosin were 104.3 (CV 0.9%), 98.72 (CV 1.6%), 95.5 (CV 2.0%) and 102.1 (CV 2.4%), 98.7 (CV 1.7%), 99.1 (CV 1.1%), respectively. The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Application

The validated method has been successfully used to quantitate the hydrochlorothiazide concentration in the human plasma samples after the administration of a single 12.5 mg oral dose of hydrochlorothiazide. The representative concentration vs time profiles of three subjects receiving a single dose of hydrochlorothiazide are presented in Fig. 8.

CONCLUSION

In summary, this is the first method described for the quantification of hydrochlorothiazide from human plasma by LC-MS/MS in negative ionization mode using MRM. The current method has shown acceptable precision and adequate sensitivity for the quantification of hydrochlorothiazide in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. Furthermore, it was utilized for the analysis of hundreds of subject samples. The method described is simple, rapid, sensitive, specific and fully validated according to commonly accepted criteria (Shah *et al.*, 1991). The cost-effectiveness, simplicity and speed of liquid–liquid extraction and sample turnover rate of 2.5 min per sample make it an attrative procedure in high-throughput bioanalysis of hydrochlorothiazide. The validated method allows quantification of hydrochlorothiazide over a range of 0.5–200 ng/mL.

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