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Short communication

Determination of hydrochlorothiazide in human plasma by liquid chromatography/tandem mass spectrometry

Fei Liu [a], Yu Xu [a,*], Shu Gao [b], Jundong zhang [b], Qingxiang Guo [a,*]

[a] *Department of Chemistry, University of Science and Technology of China, Hefei 230026, China*
[b] *Hefei Con-source Medicine Technology Corporation, Hefei 230088, China*

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Abstract

In this study, a fast and sensitive liquid chromatography/tandem mass spectrometry method for determination of hydrochlorothiazide in human plasma was developed and validated. The analyte and irbesartan, used as the internal standard, were precipitated and extracted from plasma using methanol. Analysis was performed on a Phenomenex Kromasil C_8 column with water and methanol (27:73, v/v) as the mobile phase. Linearity was assessed from 0.78 to 200 ng/mL in plasma. The analytical method proved to be applicable in a pharmacokinetic study after oral administration of 12 mg hydrochlorothiazide tablets to 20 healthy volunteers.

Keywords: Hydrochlorothiazide; Liquid chromatography; Mass spectrometry

1. Introduction

Hydrochlorothiazide, 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide -1,1-dioxide, is a diuretic and antihypertensive agent that reduces plasma volume by increasing the excretion of sodium, chloride and water and, to a lesser extend, that of potassium ion as well [1–3]. It was often used in combination with other antihypertensive drugs such as beta blockers, angiotensin-converting enzyme (ACE) inhibitors, or more recently, angiotensin II receptor blockers (ARBs) [4–10].

Numerous publications described the determination of hydrochlorothiazide concentration in plasma or urine by high-performance liquid chromatography (HPLC) with ultraviolet or electrochemical detection [10–18]. However, most of them were time consuming or not sufficiently sensitive. The coupling of HPLC and mass spectrometry has provided a useful technique for drug bioanalysis. Takubo et al. [19] reported a LC/MS/MS method for the determination of hydrochlorothiazide in rat plasma over the range 4 ∼ 1000 ng/mL. Vonaparti et al. [1] employed solid-phase extraction while Fang et al. [20] and Ramakrishna et al. [4] used liquid–liquid extraction which made the operation more complex.

The purpose of present study is to develop a fast and sensitive analytical method for the quantification of hydrochlorothiazide in human plasma. The proposed method used a relatively simple extraction procedure using methanol directly precipitate protein in combination with LC/MS/MS detection.

2. Experimental

2.1. Chemicals

Hydrochlorothiazide was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The internal standard (IS), irbesartan (99% purity), was supplied by Jiangsu Wangao Pharmaceutical Limited Company (Haimen, China). Methanol (HPLC-grade) was obtained from Merck (Darmstadt, Germany). Distilled water, prepared from demineralized water, was used throughout the study. Blank plasma was provided by The First Affiliated Hospital of Anhui Medical University (Hefei, China), and it was pooled from 20 fasted subjects.

2.2. Instrumentation

The LC/MS/MS system was comprised of a TSQ Quantum Ultra AM triple-quadrupole mass spectrometer (Thermo

* Corresponding authors Tel.: +86 551 360 0659; fax: +86 551 360 6689.
E-mail addresses: xyu@ustc.edu.cn (Y. Xu), qxguo@ustc.edu.cn (Q. Guo).

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Finnigan, USA), coupled with an electrospray ionization (ESI) source, a Finnigan Surveyor LC pump and a Finnigan Surveyor autosampler. Instrument control, data acquisition and processing were performed with Xcalibur 1.4 software (ThermoFinnigan, USA).

2.3. LC/MS/MS conditions

Hydrochlorothiazide was analyzed by a Phenomenex Kromasil C_8 (250 mm × 4.6 mm i.d., 5 μm) column (Phenomenex, USA), which was protected by a SecurityGuard C_{18}, 5 μm (4 mm × 3.0 mm i.d.) guard column (Phenomenex, USA). The mobile phase was a mixture of water and methanol (27:73, v/v), which was pumped at a flow rate of 1.0 mL/min. A post-column divert valve was used to direct HPLC eluate to a waste container in the first 2 min of the chromatographic run and afterwards to the ionization source. Mass spectrometric detection was performed in negative ion mode, using selected reaction monitoring (SRM). The signal of hydrochlorothiazide was optimized on the total ion current in MS mode, producing the following parameters: capillary temperature of 320 °C, electrospray voltage of 4.0 kV, sheath gas and auxiliary gas at the pressures (arbitrary units) of 42 and 7, respectively. At the same time, the selection of ions and the collision-induced dissociation (CID) was optimized. In MS/MS mode, the deprotonated precursor molecular ions of hydrochlorothiazide (*m/z* 295.9) and the IS (*m/z* 426.9) were chosen and fragmentized by argon with a collision gas pressure of 0.9 mTorr at a collision energy of 25 eV in both cases. The product ion of $[M-H]^-$ ions were acquired in the SRM mode at *m/z* 204.9 for hydrochlorothiazide and *m/z* 192.9 for IS, they were selected for quantification. The scan width for SRM was *m/z* 0.1, and scan time was 0.5 s. The peak width settings for both Q1 and Q3 were 0.7 u.

2.4. Preparation of standards and quality control samples

Stock solutions of hydrochlorothiazide and irbesartan were prepared in methanol at a concentration of 500 and 400 μg/mL, respectively. It was further diluted to spike the standards and Quality control (QC) samples. Calibration curves for hydrochlorothiazide were prepared by spiking blank plasma at concentrations of 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 ng/mL.

QC samples were prepared by spiking blank plasma at concentrations of 1.56, 12.5 and 100 ng/mL, representing low, medium and high concentration QC samples, respectively. QC samples were prepared independent of the standards.

2.5. Sample extraction procedure

QC samples, calibration standards and unknown plasma samples were all extracted as follows: 10 μL of IS solution (40 ng/mL irbesartan in methanol) was spiked to a 100 μL aliquot of plasma sample. The sample mixture was vortex-mixed for 30 s. Then 300 μL methanol was added to the mixture and vortex-mixed for 2 min. After centrifugation at 13,400 × *g* for 10 min to remove precipitate, 20 μL aliquot of supernatant was injected into the LC/MS/MS system for analysis.

2.6. Assay validation

The validation was carried out according to the recommendations and definitions provided by the "Guidance for Industry on Bioanalytical Method Validation" (FDA, USA) [21].

For the calibration standards, peak area ratios (the analyte/IS) were plotted against nominal plasma concentrations, and fitted by weighted ($1/x^2$) least-squares linear regression. Plasma calibration curves were prepared and assayed in triplicate on three separate days.

Accuracy and precision were assessed in conjunction with the linearity studies by determining QC samples using quintuplicate ($n=5$) preparations of spiked plasma samples at three concentration levels on three separate days. The accuracy was expressed by (mean observed concentration − spiked concentration)/(spiked concentration) × 100%, the precision was assessed in terms of the relative standard deviation (R.S.D.) of the measured concentrations. The acceptable criterion was 15% or better.

The recoveries of hydrochlorothiazide from the extraction procedure were evaluated by a comparison of the mean peak areas of QC samples at three levels to that of samples prepared by spiking extracted drug-free plasma (spike-after-extraction) samples with the same amounts of hydrochlorothiazide at the step immediately prior to chromatography.

Owing to the components of the sample matrix, signal suppression or enhancement may occur. Matrix effect was calculated by comparing peak areas of hydrochlorothiazide obtained from the spike-after-extraction samples with those from pure standard solutions at the same concentrations.

Hydrochlorothiazide stability in plasma was assessed by analyzing QC samples at concentrations of 1.56, 12.5 and 100 ng/mL, respectively, in triplicate ($n=3$), after exposure to different conditions of time and temperature. The results were compared with those for freshly prepared QC samples, and the percentage concentration deviation was calculated. For short-term stability, the plasma samples were kept at room temperature (20 °C) for 4 h and 12 h. The stability was also evaluated after storage of the plasma samples at −20 °C for 7days. The freeze/thaw stability was obtained after three freeze/thaw cycles on consecutive days.

3. Results and discussion

3.1. LC/MS/MS conditions

Hydrochlorothiazide has several polar groups; the presence of amido-group makes it very easy to produce good mass spectrometric responses in negative ionization mode. The CID of the $[M-H]^-$ ion of hydrochlorothiazide (*m/z* 295.9) and irbesartan (*m/z* 426.9) produced an abundant product ion at *m/z* 204.9 and *m/z* 192.9, respectively. The optimum collision energy was both 25 eV.

To optimize the LC system, several columns were tried: C_{18}, CN, Phenyl and C_8 columns. Of all the columns, Phenomenex Kromasil C_8 column could yield suitable retention, the best peak shape and signal-to-noise ratio leading to a lower detection limit.

In order to achieve maximum peak responses and symmetrical chromatographic peaks, mobile phases containing varying percentages of organic phase were tested. As a result, a mixture of water and methanol (27:73, v/v) was chosen as the optimized mobile phase.

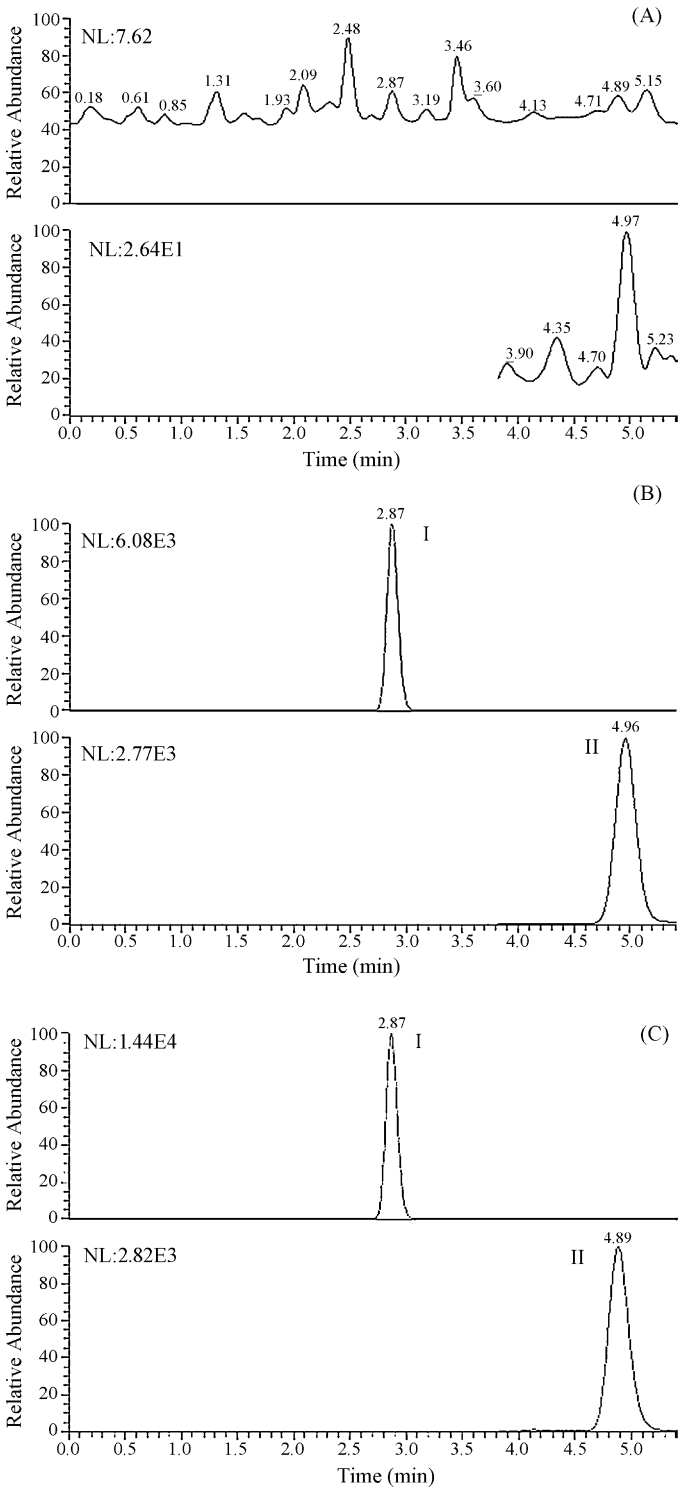

Fig. 1. SRM chromatograms for hydrochlorothiazide (I) and irbesartan (IS, II) in human plasma: (A) blank plasma; (B) blank plasma spiked with hydrochlorothiazide (25 ng/mL) and IS (4 ng/mL); (C) a plasma sample from a volunteer 8 h after an oral dose of 12 mg hydrochlorothiazide.

3.2. Specificity

For the examination on the presence of disturbing endogenous substances, human plasma from six different, drug-free subjects were tested. The specificity was examined by analyzing blank human plasma (Fig. 1 (A)) and spiked with hydrochlorothiazide (25 ng/mL) and the internal standard (4 ng/mL) (Fig. 1 (B)). As shown in Fig. 1 (A), no significant peaks from endogenous substances were seen with the elution of hydrochlorothiazide and IS. Fig. 1 (C) showed the chromatogram for a volunteer plasma sample obtained at 8 h after an oral dose of 12 mg hydrochlorothiazide. Due to the relatively short chromatographic run time (5.5 min per run) and simple sample preparation procedure, a sample throughput of 150 per day was routinely achieved.

3.3. Linearity, precision and accuracy

The calibration curves were linear over the concentration range of 0.78 ~ 200 ng/mL for the analyte. Correlation coefficients generated by linear regression with a $1/x^2$ weighting factor ranged from 0.996 to 0.999.

The lower limit of quantification (LLOQ) was confirmed to be 0.78 ng/mL, at which the calculated accuracy and precision were below 20%.

Table 1 enlisted the precision and accuracy results of the validation for hydrochlorothiazide. Intra-assay precision and accuracy of the assay were measured by analyzing spiked samples prepared the same day in quintuplicate ($n = 5$) at each QC level. Precision ranged from 2.26 to 9.92% and relative error was not more than ±11.8%. Inter-assay precision and accuracy were accessed over three days. Both precision and accuracy deviation values were within the acceptable criterion (<±15%), which confirmed the method was accurate and precise enough.

3.4. Recovery and matrix effect

The results of the recovery study for hydrochlorothiazide were found to be 81.86 ± 3.20%, 94.76 ± 9.13% and 90.07 ± 6.40% at concentrations of 1.56, 12.5 and 100 ng/mL, respectively ($n = 3$).

Matrix effects were investigated by analysis of spike-after-extraction samples with pure standard solutions at the same concentrations and the results is 92.68~94.29% for hydrochlorothiazide. Thus, ion suppression or enhancement from plasma matrix was negligible for this method.

3.5. Stability

The stability studies indicated acceptable variation in drug concentration over a span of 12 h at room temperature. Upon storage of samples being kept frozen for a week and during three freeze/thaw cycles, reliable stability behavior of hydrochlorothiazide was observed (all within ± 14.1%). The stability data of hydrochlorothiazide in plasma under different temperature and time conditions are given in Table 2.

Table 1
Precision and accuracy results of validation

Day of analysis		Hydrochlorothiazide concentration in human plasma (ng/mL)		
		Low QC 1.56	Medium QC 12.5	High QC 100
Day 1	Mean (ng/mL)	1.48 ± 0.13	13.04 ± 0.47	89.27 ± 3.25
	R.S.D. (%)	8.91	3.62	3.64
	R.E. (%)	−5.6	4.3	−10.7
Day 2	Mean (ng/mL)	1.67 ± 0.15	12.34 ± 0.91	89.81 ± 2.03
	R.S.D. (%)	8.95	7.37	2.26
	R.E. (%)	6.9	−1.3	−10.2
Day 3	Mean (ng/mL)	1.75 ± 0.11	12.72 ± 1.26	104.83 ± 6.91
	R.S.D. (%)	6.43	9.92	8.31
	R.E. (%)	11.8	1.8	4.8
Inter-assay S.D. (ng/mL)		0.14	0.35	8.83
Inter-assay mean (ng/mL)		1.63	12.70	94.64
Inter-assay R.S.D. (%)		8.59	2.76	9.33
Inter-assay R.E. (%)		4.4	1.6	−5.4

R.S.D.: relative standard deviation; R.E.: relative error.

3.6. Application

The developed method was applied to a pharmacokinetic study in which the concentration of hydrochlorothiazide was measured in plasma samples from 20 volunteers after single oral dose of 12 mg hydrochlorothiazide. The experimental protocol was approved by the institutional review board of the State Base for Drug Clinical Trial of Anhui Medical University (Heifei, China), and written informed consent was obtained from all participants. The drug was administrated under fasting condition.

The profile of the mean plasma concentration versus time is illustrated in Fig. 2. The maximum plasma concentration (C_{max}) was 68.82 ± 26.31 ng/mL; the area under the curve ($AUC_{0\rightarrow48}$) was 421.06 ± 154.66 ng·h/mL; the time to maximum plasma concentration (T_{max}) was 2.0 ± 0.6 h; and the half-life ($t_{1/2}$) was 8.98 ± 2.41 h. The present method proved to be applicable in the pharmacokinetic study.

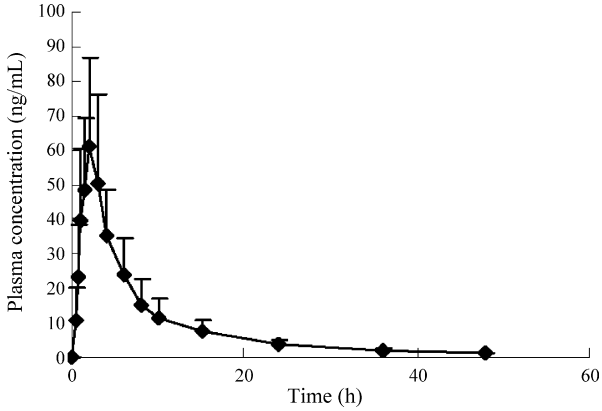

Fig. 2. Mean plasma concentration-time profile of hydrochlorothiazide for 20 healthy volunteers after a single 12 mg oral dose. (n = 20, mean value and S.D. are plotted).

Table 2
Stability data for assay of hydrochlorothiazide in human plasma (n = 3) under various storage conditions

Storage conditions	Conc. Spiked (ng/mL)	Conc. Found (ng/mL)	R.S.D. (%)	R.E. (%)
4 h at R.T.	1.56	1.48	10.27	−5.1
	12.5	13.54	6.10	8.3
	100	99.79	3.39	−0.2
12 h at R.T.	1.56	1.34	7.11	−14.1
	12.5	13.47	0.32	7.8
	100	92.79	6.57	−7.2
Freezer for 7 days	1.56	1.35	6.49	−13.5
	12.5	11.64	10.67	−6.9
	100	88.67	4.01	−11.3
Three freeze/thaw cycles	1.56	1.39	4.22	−10.9
	12.5	12.78	4.07	2.2
	100	90.53	5.48	−9.5

R.S.D.: relative standard deviation; R.E.: relative error; R.T.: room temperature.

4. Conclusion

We have described a simple, rapid LC/MS/MS assay for the quantification of hydrochlorothiazide in human plasma, which showed acceptable precision and adequate sensitivity. Hence, it can be easily adopted for the high-throughput routine bioanalysis of hydrochlorothiazide.

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