Sensitive and selective liquid chromatography–electrospray ionization tandem mass spectrometry analysis of hydrochlorothiazide in rat plasma

Takatoshi Takubo*, Hiromasa Okada, Mikio Ishii, Ken-ichi Hara, Yasuyuki Ishii

Drug Metabolism, Tsukuba Research Institute, Banyu Pharmaceutical Co. Ltd., 3, Okubo, Tsukuba, Ibaraki 300-2611, Japan

Abstract

A sensitive and selective method for the determination of hydrochlorothiazide (HCTZ) concentrations in rat plasma was developed using high performance liquid chromatography–electrospray ionization tandem mass spectrometry (LC–MS/MS). An aliquot of plasma (50 μl) was mixed with the solution of internal standard, hydrofluorothiazide (HFTZ), and extracted with *tert*-butyl methyl ether. The reconstituted extract was applied to the LC–MS/MS system with a reversed phase C8 column and eluted with distilled water/acetonitrile (85/15, v/v). To enhance negative ionization of HCTZ and HFTZ in the multiple reaction monitor (MRM), the solution consisting of acetonitlile/1% (v/v) ammonia solution (95/5, v/v) was delivered after column separation. This additional technique, so-called the post-column addition, increased sensitivity of HCTZ and HFTZ about 500- and 200-fold, respectively. The calibration curve showed good linearity ($r = 0.999$) over the range of 4–1000 ng/ml. Acceptable accuracy (100.8–113.1%) and precision (0.28–16.4%) were confirmed in the intra- and the inter-day analyses. It is indicated that this LC–MS/MS method is useful for pharmacokinetic studies of HCTZ in small animals, because it enabled the serial determination of plasma level of HCTZ in rats.
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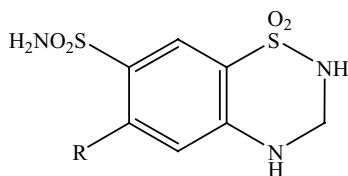*Keyword:* Hydrochlorothiazide

1. Introduction

Hydrochlorothiazide (HCTZ, Fig. 1) is a common diuretic and anti-hypertensive agent, which reduces plasma volume by increasing the excretion of sodium, chloride and water. The decrease in plasma volume, however, causes counter-regulatory stimulation of the rennin–angiotensin system and the sympathetic nervous system [1,2]. This interaction between diuretics and the rennin–angiotensin system leads to combination therapy, for example, low-dose diuretics enhance the response to angiotensin II AT-1 receptor blockers. Meanwhile, AT-1 receptor blockers are expected to suppress the adverse effects of diuretics [2,3]. In the case of a combination of HCTZ and losartan, a highly selective AT-1 receptor antagonist, clinical studies demonstrated anti-hypertensive efficacy and tolerability of the combination therapy in patients with moderate-to-severe essential hypertension who would be inadequately controlled by mono-drug therapy [3,4].

To investigate more detailed mechanism of the interaction between HCTZ and AT-1 receptor blockers, it would be necessary to monitor the plasma concentration of HCTZ in small animals such as rats. However, few methods for the determination of HCTZ in plasma collected from small animals or from humans have been reported and none of them could monitor the plasma concentration-time profile of HCTZ in rats. Kuo et al. reported the column-switching HPLC method with ultraviolet spectrometry (LC-UV) for the determination of HCTZ in rat, dog and human plasma [5]. Their method required 200–300 μl of rat plasma to obtain the quantification range from 25 to 2000 ng/ml. Niopas and Daftsios determined HCTZ in human plasma at the range from 5 to 80 ng/ml by the LC-UV method using 1 ml sample [6]. These LC-UV methods would not be applicable to monitor the plasma concentration–time profile of HCTZ in rats, because the reasonable volume of plasma obtained from a serially bled rat would be less than 100 μl per sample, without exsanguinations.

* Corresponding author. Tel.: +81-29-877-2353;
fax: +81-29-877-2352.
E-mail address: takatoshi_takubo@merck.com (T. Takubo).

R=Cl: HCTZ, R=F: HFTZ

Fig. 1. Chemical structure of hydrochlorothiazide (HCTZ) and an internal standard, hydrofluorothiazide (HFTZ).

Recently, Franolic et al. reported for the first time that HCTZ and its impurities in drug substances were characterized by the combination of HPLC and electrospray ionization MS (ESI–MS) in the negative ion mode using multiple reaction monitoring (MRM) [7]. In this study, a sensitive and selective analytical method for the determination of HCTZ in rat plasma was developed using LC–MS/MS technique. The analytical method of Franolic et al. is not highly sensitive to determine HCTZ, and therefore, the post-column addition technique was adopted to enhance the negative ionization of HCTZ and raise the sensitivity for quantification of HCTZ. The purpose of this study was to monitor the plasma concentration-time profile of HCTZ in rats administered at a low dose of HCTZ per os.

2. Experimental

2.1. Materials

Hydrochlorothiazide (Fig. 1, 99.6%) was supplied by Merck & Co. Inc. (Rahway, NJ) and hydrofluorothiazide (HFTZ, Fig. 1) was purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile of HPLC grade was obtained from Wako Pure Chemical Industries (Osaka, Japan) and ammonium solution was from Kanto Chemical Co. Inc. (Tokyo, Japan). All other chemicals were of reagent grade.

2.2. Extraction procedure

An aliquot of rat plasma (50 μl) was mixed with HFTZ (50 ng) dissolved in methanol (30 μl) and 420 μl of 80 mM carbonate buffer solution (pH 9.6). This mixture was applied to Extrelut® NT1 column (Merck KGaA, Darmstadt, Germany), and eluted with 6 ml of *tert*-butyl methyl ether after standing for 10 min at room temperature. This solution was dried under nitrogen gas flow at 40 °C, and then reconstituted in 150 μl of 10% (v/v) aqueous acetonitrile. A 20 μl aliquot of this sample was injected to the LC–MS/MS system.

2.3. LC–MS/MS analysis

The LC–MS/MS system consisted of two Quaternary Pumps, a Vacuum Degasser, an Autosampler and a Thermostatted Column Compartment (Hewlett Packard SERIES

1100: Yokogawa Analytical Systems, Tokyo, Japan) and a MDS SCIEX API 300 LC/MS/MS System with a Turbo Ionspray® interface (Applied Biosystems, Tokyo, Japan). The analytical column was a CAPCELL PAK C8 (UG 120, 5 μm, 1.5 mm × 150 mm: Shiseido, Tokyo, Japan) column temperature of 50 °C. The mobile phase for column separation was distilled water/acetonitrile (85/15, v/v) with a flow rate of 100 μl/min. The solution of acetonitrile/1% (v/v) ammonia solution (95/5, v/v) was additionally pumped at a flow rate of 100 μl/min after the column separation as a post-column addition. Electrospray ionization was performed in the negative ion mode heated nebulizer with a temperature of 425 °C and ionspray voltage of −4200 V. The nebulizer, curtain and collision activated dissociation gas was set to 10, 12 and 4, respectively. LC–MS/MS chromatograms were acquired in MRM of the transition of the deprotonated molecular ion of m/z 296 to the product ion of m/z 205 for HCTZ (MW 297) and the deprotonated molecular ion of m/z 330 to the product ion of m/z 239 for HFTZ (MW 331). The declustering potential, collision energy and collision cell exit potential were set −34, −28 and −20 V for HCTZ and −38, −30 and −20 V for HFTZ. The installed software (PE-SCIEX Analyst Software, version 1.1) was used for the quantification of HCTZ.

2.4. Method validation

HCTZ was dissolved with methanol and blank plasma pooled from thirty rats was used for this method validation. To prepare a calibration standard, the plasma was spiked with the methanol solution of HCTZ at the final concentration of 4, 16, 40, 160, 400 and 1000 ng/ml. The calibration curve was constructed by weighted ($1/y^2$) least-squares linear regression analysis of the peak area ratios of HCTZ to HFTZ and the concentrations of HCTZ. The precision was expressed as relative standard deviation, and the accuracy was calculated by the following equation:

$$\text{accuracy (\%)} = \frac{\text{mean observed concentration}}{\text{spiked concentration}} \times 100.$$

The precision and accuracy of intra-day analysis were evaluated from the observed concentrations of spiked HCTZ in five replicate analyses at 4, 160 and 1000 ng/ml. The inter-day reproducibility was evaluated from the observed concentrations of spiked HCTZ in triplicate analyses at 16 and 400 ng/ml on each of 4 days. Stability of HCTZ in rat plasma at room temperature, below −70 °C, after three freeze and thaw cycles, and in the reconstituted extract set in the Autosampler was also investigated in triplicate analyses at 16 and 400 ng/ml. To confirm potential interference of endogenous compounds extracted from rat plasma, an aliquot of the blank plasma (50 μl) was analyzed according to the methods as described above. Matrix effect was investigated using analyte standard (25 g HCTZ and 50 ng HFTZ in 150 μl 10% (v/v) aqueous acetonitrile) and blank plasma collected from six rats separately. Peak area of HCTZ and

HFTZ was compared in the LC–MS/MS analysis of the standard alone and the standard spiked with each extract of individual blank plasma prepared by the above procedure.

2.5. Animal study

Nine week old male Crj: CD(SD) IGS BR rats (Charles River Japan Inc., Kanagawa Japan) were used in this study. A polyethylene cannula was placed into the left carotid artery of each rat under pentobarbital anesthesia 3 days before dosing. After fasting overnight, rats ($n = 3$) received HCTZ suspended in 0.5% (w/v) methylcellulose solution as a single oral dose equivalent to 3.75 mg/kg body-weight. Aliquots of blood (250 μl) were serially collected from the cannula using heparinized capillaries at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h post dosing. Plasma samples were obtained from the blood by centrifugation (12,000 × g, 10 min, 4 °C) and stored below −70 °C until the analysis. This animal protocol was approved by the Institutional Animal Care and Use Committee of the Banyu Pharmaceutical Co. Ltd. Concentrations of HCTZ in the plasma samples were determined by using the method as described above. Pharmacokinetic parameters of HCTZ such as an apparent elimination half-life ($t_{1/2}$) and area under the plasma concentration versus time curve (AUC) were calculated using commercial software (WinNonlin Professional, version 3.3 (Pharsight Corporation, Mountain View, CA)).

3. Results and discussion

3.1. Condition of LC–MS/MS analysis

To determine the concentration of HCTZ in rat plasma, we modified the LC–MS method reported by Franolic et al. [7]. Our LC condition adopted a reversed phase C8 analytical column with a 15% (v/v) aqueous acetonitrile as a mobile phase, instead of a C18 analytical column and gradient elution. The ESI-MS was performed in the negative ion mode following their reported condition. Although HCTZ and HFTZ were well separated in this LC condition, it was obvious that the sensitivity of HCTZ in this LC–MS analysis was not sufficient to monitor the plasma level of HCTZ in respective rats using less than 100 μl plasma sample. To improve the sensitivity of the HCTZ assay, the effects of various solvents on the negative ionization of HCTZ were examined by the direct infusion of the standard solution. The sensitivity of the HCTZ assay increased when HCTZ was infused with a solution containing aqueous ammonia or with a higher concentration of acetonitrile. However, HCTZ was not retained on both C8 and C18 analytical columns with these mobile phases so that separation of HCTZ from endogenous components was not expected in these conditions (data not shown).

Several recent reports indicated that addition of various solvents after column separation, the so-called post-column addition technique, improved sensitivity for quantification in LC–MS/MS analysis of some compounds, such as a calcium antagonist, oligonucleotides, bacteriochlorophylls and catecholamines [8–11]. Greig and Griffey reported similar enhanced ESI-MS detection of oligonucleotides in negative ion mode by the addition of organic bases using flow injection and direct infusion [12]. The mechanism of enhancement of ionization has not been clarified, though they found that a 1:1 co-addition of a strong base (triethylamine or piperidine) plus a weak base (imikazole) drastically reduced Na^+ and K^+ adducts, resulting in improved sensitivity for oligonucleotides [12]. Based on these finding, we attempted to use the post-column addition technique in this LC–MS/MS analysis of HCTZ and we infused acetonitrile solution containing aqueous ammonia into the line after column separation.

Fig. 2 shows peaks of HCTZ and HFTZ in MRM chromatogram with the post-column addition of the same solution as the mobile phase (Fig. 2a) and of acetonitrile/1% (v/v) ammonia solution (95/5, v/v) (Fig. 2b). The peak shapes of both HCTZ and HFTZ were not affected by the addition of these solutions after column separation. In both conditions, HCTZ was well separated from HFTZ and the retention time of HCTZ and HFTZ was about 6 and 10 min, respectively. The peak areas of HCTZ and HFTZ were increased approximately 500- and 200-fold, respectively, by the post-column addition of basic acetonitrile solution. These findings suggested that sensitive determination of HCTZ concentrations by LC–MS/MS analysis could be achieved by the post-column addition of organic base solution for enhanced negative ionization of HCTZ.

3.2. Method validation

Typical MRM chromatograms of extracts prepared from an aliquot (50 μl) of blank plasma pooled from thirty rats and plasma spiked with 4 ng/ml of HCTZ are shown in Fig. 3. The HCTZ peak of the MRM chromatogram was obviously distinguished from the background noise. No interference by endogenous compounds was observed at the position of the HCTZ peak. From these results, the lower limit of quantification was determined to be 4 ng/ml. Peak area of HCTZ and HFTZ in the LC–MS/MS analysis of the standard spiked with each extract of blank plasma was accounted for 101.9 ± 1.86 and $100.7 \pm 1.08\%$ (mean ± S.D.) of their peak area in the LC–MS/MS analysis of the standard alone, respectively, suggesting no considerable matrix effect on the LC–MS/MS analysis of HCTZ and HFTZ.

A calibration curve constructed by weighted ($1/y^2$) least-squares linear regression analysis of the peak area ratios of HCTZ to HFTZ, and the concentrations of HCTZ indicated acceptable linearity ($r = 0.999$) at plasma concentrations in the range of 4–1000 ng/ml. Precision and accuracy of determined concentrations in intra-day analysis are shown in Table 1. At three concentrations of HCTZ including the lower and upper limit of quantification, the accuracy and precision ranged from 106.8 to 108.9% and
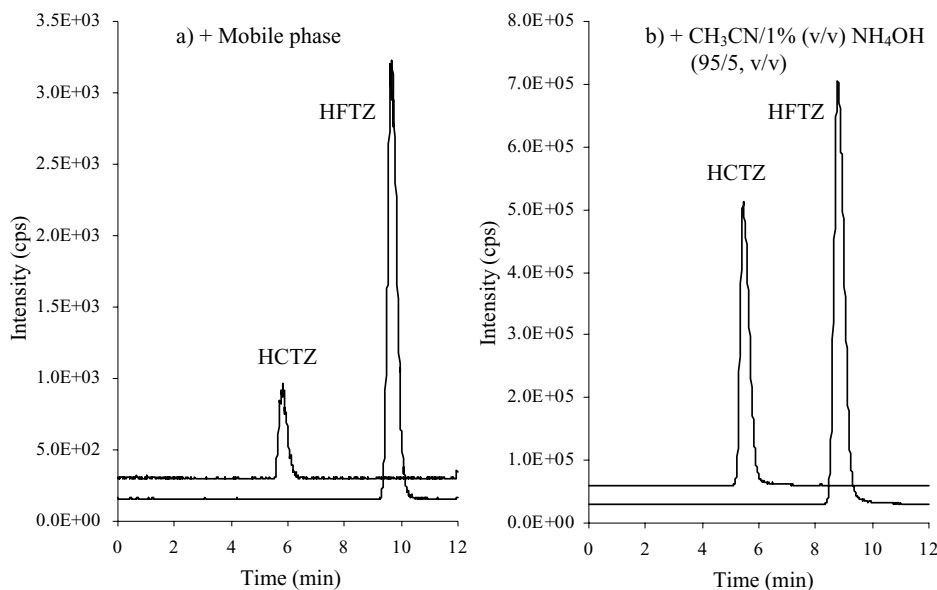
Fig. 2. Effect of post-column addition on MRM chromatogram of HCTZ and HFTZ (20 ng each) in LC–MS/MS analysis.
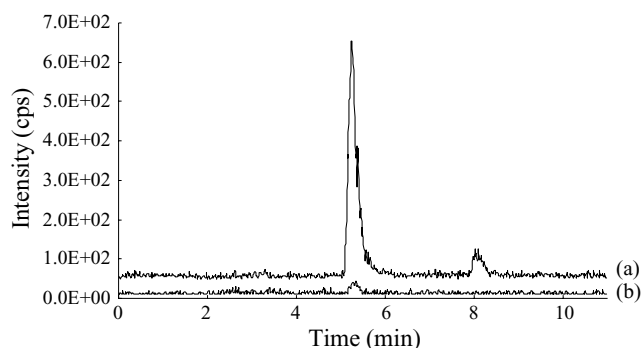

Fig. 3. Typical MRM chromatogram of the extracts from rat plasma spiked with HCTZ (4 ng/ml) (a) and rat blank plasma (b).

0.48 to 16.4%, respectively. In the inter-day analysis, the accuracy and precision at two concentrations of HCTZ in a 4 day analysis ranged from 100.8 to 113.1% and 0.28 to 3.01%, respectively (Table 2). These results indicate that quantification of HCTZ concentration in rat plasma with acceptable accuracy and precision is established using this LC–MS/MS analysis.

The determined concentration of HCTZ in rat plasma was accounted for 99.3 ± 2.73 and $103 \pm 0.24\%$ (mean $\pm$ S.D.) of

initial concentration, stored for 6 h at room temperature, and was accounted for 105 ± 2.08 and $98.3 \pm 2.96\%$ (mean $\pm$ S.D.) of initial concentration, stored for 11 days below $-70\,^{\circ}$C, at 16 and 400 ng/ml, respectively, indicating that HCTZ is stable in rat plasma at room temperature for at least 6 h and below $-70\,^{\circ}$C for at least 11 days. In three freeze and thaw cycles, determined concentrations of HCTZ in rat plasma ranged from 96.7 to 103% of initial concentration at the

Table 2
Inter-day accuracy and precision for the determination of HCTZ in rat plasma

Spiked concentration (ng/ml)	Day	Determined concentration (ng/ml)	Accuracy (%)	Precision (%)
16	1	17.3 ± 0.23	108.3	1.33
	2	17.4 ± 0.35	108.5	2.02
	3	17.9 ± 0.15	111.7	0.85
	4	18.1 ± 0.36	113.1	1.99
400	1	410 ± 5.0	102.6	1.23
	2	409 ± 1.2	102.2	0.28
	3	414 ± 1.7	103.5	0.42
	4	403 ± 12.1	100.8	3.01

Results are expressed as mean ± S.D. ($n = 3$).

Table 1
Intra-day accuracy and precision for the determination of HCTZ in rat plasma

Spiked concentration (ng/ml)	Determined concentration (ng/ml)	Accuracy (%)	Precision (%)
4	4.34 ± 0.71	108.6	16.4
160	174 ± 0.84	108.9	0.48
1000	1068 ± 13.2	106.8	1.23

Results are expressed as mean ± S.D. ($n = 5$).

Table 3
Freeze and thaw stability of HCTZ in rat plasma

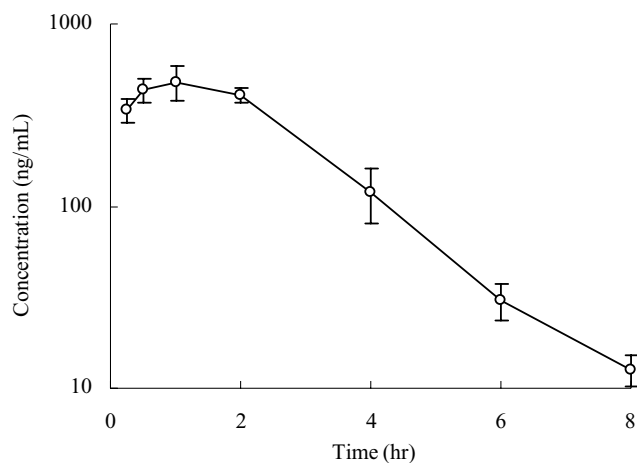| Freezing and thawing times	Measured concentration ratio (%)	
	16 ng/ml	400 ng/ml
0	100	100
1	103 ± 0.33	98.9 ± 0.78
2	102 ± 1.53	99.2 ± 0.78
3	103 ± 1.45	96.7 ± 1.39

Results are expressed as mean ± S.D. ($n = 3$).

Fig. 4. Mean (±S.D.) plasma concentration–time profile of HCTZ in three rats received an oral dose of HCTZ (3.75 mg/kg).

two concentrations (Table 3) and it is suggested three freeze and thaw cycles are fully tolerable for the determination of HCTZ concentration in rat plasma. The determined concentration of HCTZ in the reconstituted extract stored for 37 h at 4 °C in the Autosampler was accounted for 98.3 ± 2.21 and $95.2 \pm 1.74\%$ (mean ± S.D.) of initial concentration at 16 and 400 ng/ml, respectively, indicating that HCTZ in reconstituted extract is stable for at least 37 h in the Autosampler.

3.3. Pharmacokinetic study

This LC–MS/MS method was applied to determine the plasma concentration of HCTZ in three male rats that received a single oral dose of HCTZ (3.75 mg/kg). Plasma samples were serially collected from rats up to 24 h post-dosing. In all three rats, the plasma concentrations of HCTZ were higher than the lower limit of quantification (4 ng/ml) up to 8 h post-dosing (Fig. 4) and HCTZ was hardly detectable at 24 h post-dosing. The maximum plasma concentration (C_{max}) was observed at 1.17 ± 0.76 h post-dosing and accounted for 514 ± 78 ng/ml (mean±S.D.). Pharmacokinetic parameters, $t_{1/2}$ and $AUC_{0–8}$, were also estimated in rats. The mean (±S.D.) value of $t_{1/2}$ was estimated for 1.26 ± 0.21 h, indicating rapid clearance of HCTZ in rats. The mean (±S.D.) value of $AUC_{0–8}$ was calculated at 1.47 ± 0.10 μg h/ml. We cannot find out any literature indicating such pharmacokinetic data of HCTZ in rats. Further pharmacokinetic data of HCTZ in rats or in other small animals that received HCTZ alone or with other agents would be easily estimated using this LC–MS/MS analysis and these data might help clarify the detailed mechanism of pharmacological and toxicological interaction between HCTZ and AT-1 receptor blockers. In addition to providing higher sensitivity using a smaller plasma volume, the LC–MS/MS analysis could also be used to monitor plasma concentration of HCTZ in clinical samples.

4. Conclusion

A sensitive and selective assay for HCTZ concentrations in rat plasma was developed using the LC–MS/MS technique with a post-column addition of organic base solution. This is the first application of a post-column addition technique to LC–MS/MS analysis of HCTZ. Rat plasma pharmacokinetic parameters for HCTZ were precisely established based on the results generated using this methodology.

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