



## Rapid Determination of Hydrochlorothiazide in Human Plasma by High Performance Liquid Chromatography-Tandem Mass Spectrometry

Carlos E.M. SOUSA, Danilo C.G. BEDOR \*, Talita M. GONÇALVES, Virma L.S. RAMOS,  
Andre L.M. CARVALHO, Miracy M. ALBUQUERQUE & Davi P. SANTANA

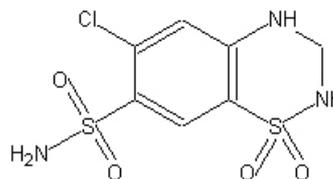
*Núcleo de Desenvolvimento Farmacêutico e Cosmético, Rua Professor Arthur de Sá s/n,  
Cidade Universitária, CEP 50740-520, Recife, Pernambuco, Brasil.*

**SUMMARY.** This paper describes a rapid (2.0 min) and sensitive (LLOQ 5 ng/mL) analytical method for the quantitation of hydrochlorothiazide (HCTZ) in human plasma. The method is based on High-performance Liquid chromatography-tandem mass spectrometry (LC-MS/MS) using chlortalidone as internal standard (I.S.). Sample preparation involved liquid-liquid extraction with methyl tert-butyl ether. The chromatographic separation was achieved on a monolithic C18 (50 x 4,6 mm) reversed-phase column and a mobile phase containing acetonitrile/water (80:20 v/v, add 5% isopropyl alcohol), in isocratic conditions. The target analytes were transferred into a triple quadrupole mass spectrometer equipped with an electrospray ionization source for mass detection. The ion transitions selected for MRM detection were:  $m/z$  296.10 > 204.85 and 337.13 > 189.77 for HCTZ and I.S., respectively. The assay was linear in the concentration range of 5–400 ng/mL. The mean recovery for HCTZ was 80.46%. Intra- and inter-day precision (Relative Standard Deviation) were < 10.3 % and < 11.7%, respectively and the accuracy (Relative Error) was in the range  $\pm$  4.54%, the accuracy was evaluated by the ratio between concentration found/nominal concentration. The method was successfully applied to a single oral dose pharmacokinetics study in 26 healthy human volunteers.

### INTRODUCTION

Hydrochlorothiazide (6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfo-namide -1,1-dioxide, HCTZ) (Figure 1), is a diuretic and anti-hypertensive agent that reduces plasma volume by increasing the excretion of sodium, chloride and water and, to a lesser extent, that of potassium ion as well<sup>1-4</sup>. Hydrochlorothiazide is one of the oldest thiazide diuretics, often prescribed in combination with other antihypertensive drugs such as beta blockers, angiotensin-converting enzyme inhibitors, or angiotensin II receptor blockers<sup>4</sup>.

A number of methods have been employed for the analysis of hydrochlorothiazide concentration in plasma or urine by high performance liquid chromatography (HPLC) with ultraviolet or electrochemical detection<sup>5-7</sup>. 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<sup>2,8,9</sup>. Recently, reported for the first time that HCTZ and its impurities in drug



**Figure 1.** Chemical structure of hydrochlorothiazide (HCTZ).

substances were characterized by the combination of HPLC and mass spectrometry<sup>10</sup>.

The proposed methods were useful for the resolution of band overlapping in quantitative analysis. The present work presents simple, rapid and sensitive methods for determination of HCTZ in human plasma, using a relatively simple liquid-liquid extraction procedure using in combination with LC-MS/MS analysis.

### MATERIALS AND METHODS

#### *Chemicals and reagents*

Hydrochlorothiazide reference standard was acquired from the Instituto Nacional de Controle

**KEY WORDS:** Hydrochlorothiazide, LC-MS/MS, Pharmacokinetics.

\* Author to whom correspondence should be addressed. E-mail: danilo.bedor@nudfac.com.br

de Qualidade em Saúde (INCQS, Rio de Janeiro, Brazil) and clortalidone (internal standard, I.S.) from the United States Pharmacopeia (Rockville, MD, USA). HPLC-grade isopropyl alcohol, methyl tert-butyl ether (MTBE) and acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was purified using a MilliQ® system from Millipore (Molsheim, France). Clorana® (SANOFI-SYNTHELABO) and the formulation test 50 mg (hydrochlorothiazide) tablets were purchased from the local pharmacy.

### **Instrumentation**

High performance liquid chromatography was done using a chromatograph composed of two pumps (LC 10ADvp), a column oven (CTO 10Avp), an autosampler (SIL 10ADvp), and a system controller (SCL 10Avp), Shimadzu (Kyoto, Japan). The LC equipment was connected to a Micromass Quattro LC system (Milford, USA) For sample extraction a Jouan M23i refrigerated centrifuge (St. Herblaim, França) was used. Samples were stored at -70 °C REVCO (Ascheville, NC, USA) freezer until analysis.

### **Chromatography conditions**

For the LC optimization some analytical columns were evaluated. Reversed phase C18 (Gemini 50 x 2.0 mm, 5 µm), reversed phase C18 (ACE, 50 x 4.6, 5µm) and reversed phase monolithic (Phenomenex, Onix 50 x 4.6 mm). The mobile phase was achieved by varying the percentage of organic solvent (methanol or acetonitrile) for a short analytical time, the best compromise between separation efficiency, peak cheap and stability of the MS signal.

### **MS/MS conditions**

The LC equipment was operated with negative electrospray ionization (ESI-) interface source. The mass spectrometric parameters (Cone voltage, collision energy, source temperature, desolvation gas, multiplier detection) were optimized to obtain maximum sensitivity at unit resolution. The multiple reaction monitoring (MRM) detection mode was employed to hydrochlorothiazide ( $m/z$  296.10 > 204.85) and I.S. ( $m/z$  337.13 > 189.77) parent and daughter ion fragments, respectively, with dwell time set at 0.5 s for each transition.

### **Preparation of working solutions and quality control standards**

The stock solutions of hydrochlorothiazide and I.S. (at 1.0 mg.mL<sup>-1</sup>) were prepared by dissolving the substance in acetonitrile:water (1:1 v/v). The I.S. working solution was also pre-

pared in acetonitrile:water (1:1 v/v) at a concentration of 2500 ng.mL<sup>-1</sup>.

### **Sample preparation**

Plasma samples (250 µL) were transferred to a 2 mL polypropylene vial to which internal standard (25 µL, 2500 ng.mL<sup>-1</sup>) and 1.5 mL of MTBE was added to the mixture and vortex-mixed for 1 min. The samples were centrifuged at for 5 minute at 3000 x g. The aqueous layer discarded. The organic phase was transferred to 2 mL glass vials and the solvent was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was redissolved in 250 µL of acetonitrile:water (1:1 v/v), of which 200 µL was transferred into 250 µL glass vials and placed in the autosampler for analysis. The injection volume was 40 µL.

### **Method validation**

Quantitation was based on determination of relationship between HCTZ peaks areas and I.S. peaks areas. Selectivity was evaluated by extracting plasma samples of plasma from six different volunteers, including a lipemic and hemolysed plasma. Recoveries of HCTZ at the three QC concentrations and I.S. at 2500 ng.mL<sup>-1</sup> were determined by comparing peak areas of spiked plasma samples with the peak area in solutions (acetonitrile/water 1:1 v/v) prepared with the same nominal concentration. For precision (as relative standard deviation, R.S.D.) and accuracy (as relative error, R.E.) studies, samples were prepared at three QC with 6 replicates each, and were analysed in the same day (intra-day precision and accuracy), and analysed in 3 consecutive days (inter-day precision and accuracy). The stability of the solutions and plasma samples was also evaluated during method validation. HCTZ stock solutions were analysed at two QC levels (low and high QC, corresponding to 15 and 320 ng.mL<sup>-1</sup>, respectively) both recently prepared or after 7 days stored at 4 °C. The stability of HCTZ was also evaluated in post-extracted samples kept in the autosampler at room temperature (23 °C) for 6 or 24 h, as well as in plasma samples kept at -70 °C for 72 days and after being submitted to 3 freeze-thawing cycles (24 h each cycle). All samples described above were compared to freshly prepared HCTZ samples at the same concentration level. The validation follows the ANVISA guideline for bioanalytical methods.

### **Application of method**

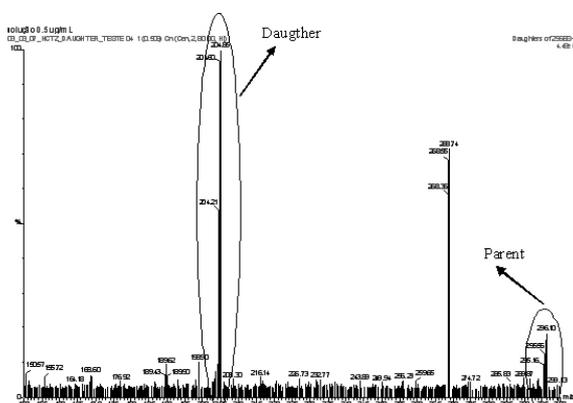
The method was applied to a single oral dose study of HCTZ (50 mg tablet) in 26 healthy

volunteers who were signed the consent before clinical trial was approved by the ethics committee of Federal University of Pernambuco. Blood samples (8 mL) were collected by venepuncture before and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 12, 24, 36 and 48 h after dosing. Following centrifugation for 5 min at 3000 x g, plasma samples were stored in polypropylene cryogenic tubes at -70 °C until analysis.

## RESULTS AND DISCUSSION

### LC- MS/MS conditions

Chromatographic separation was achieved using a Onix C18 (Monolithic) column (Phenomenex, Torrance, CA, EUA) with 50 x 4,6 mm. The mobile phase consisted of acetonitrile and water (80:20, v/v), add 5% Isopropyl alcohol which was filtered, degassed and pumped at a flow rate of 1.0 mL/min. The column oven was set at 40 °C and the injected volume was 40 µL. Retention time for HCZT and clortalidone were 0.9 min for both compounds and analytical run was 2.0 min (Fig. 3). Quantitation of hydrochlorothiazide was done by measuring the response (area) of hydrochlorothiazide in relation to the response of clortalidone (I.S.). The isopropyl alcohol was used for the best asymmetry factor. Figure 2 shows HCTZ negative ion electrospray mass spectra with parent and daughter ion (m/z 296.10 > 204.85) the mass transition monitored in MRM mode for I.S. was m/z 337.13 > 189.77. The source temperature was optimized at 110 °C, desolvation temperature was 400 °C, and desolvation gas flow was 390 L/h. The capillary voltage was set at 2.9 kV, while optimized cone voltage values for HCTZ and I.S. were 36 V and 30 V, respectively. The collision energy was optimized for HCTZ was 23 V and 18 V for I.S. The multiplier was set at



**Figure 2.** Mass spectra of HCTZ with daughter and parent ion.

900V and argon was used as the collision gas at a pressure of  $1.88 \times 10^{-3}$  psi in the collision cell.

### Method validation

#### Selectivity

The method demonstrated excellent chromatographic specificity with no endogenous or metabolite interferences at the retention times for HCTZ and clortalidone (Fig. 3). Chromatograms of extracted blank human plasma containing low (5 ng/ml) and high (400 ng/ml) concentrations of HCZT indicated good detector response and baseline resolution for HCTZ and I.S. with an analytical run time of 2 min. The cross talk test shows not interference between channels of HCTZ and I.S.. The carry over test shows not interference between samples of an analysis sequence.

#### Linearity, precision, accuracy and recovery

The calibration curves for hydrochlorothiazide were prepared in human plasma at concentrations of 5, 10, 30, 80, 100, 200, 300 e 400 ng.mL<sup>-1</sup>. Quality control (QC) samples were also prepared in human plasma at the following concentrations: 15, 190 e 320 ngmL<sup>-1</sup> (low, medium and high concentration).

Good linearity was obtained by the linear regression ( $y = 0.00645298 \pm 0.00117252 x + 0.00849646 \pm 0.00553558$ ) in the concentration range of 5 – 400 ng/mL with a mean correlation coefficient of  $0.99540 \pm 0.00355$  (n= 3 analytical runs, Table 1). The LLOQ was 5 ng/mL. The recoveries of HCTZ and I.S. using liquid-liquid extraction with MTBE were 80.46% and 76.35%, respectively.

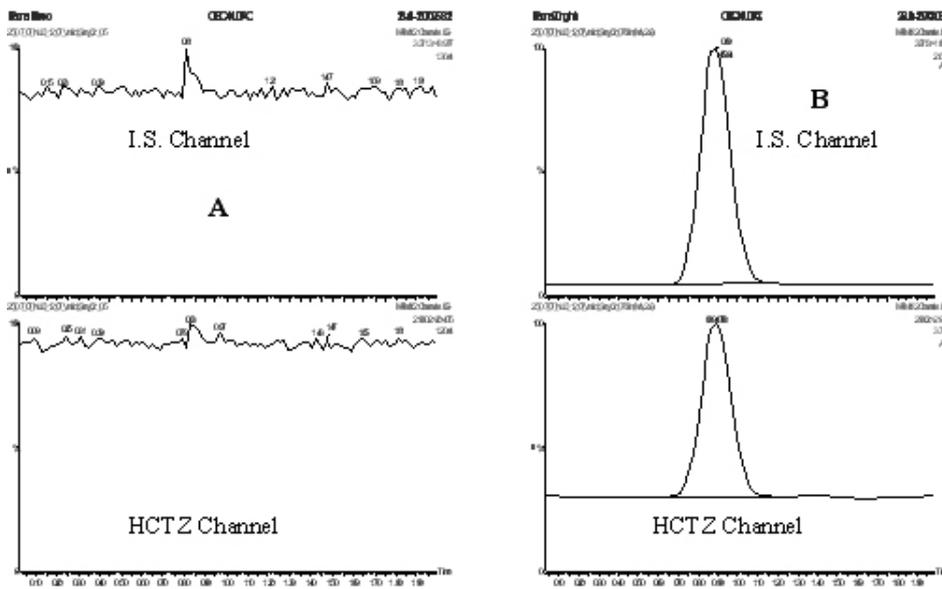
Matrix effects were investigated by analysis of spike-after-extraction samples with pure standard solutions at the same concentrations. Thus, ion suppression or enhancement from plasma matrix was negligible for this method. Intra- and inter-day precision and accuracy results (Table 2) gave satisfactory results in that R.S.D. < 11.70% and R.E. < 4.54%.

#### Stability studies

The stability data of HCTZ in plasma under different temperature, time conditions and freeze-thaw was demonstrated as the calculated concentrations for the controls did not significantly decrease over the course of the study (Table 3).

#### Pharmacokinetic study

The developed method was successfully applied to a pharmacokinetic study in which the



**Figure 3.** Representative chromatograms of (A) blank plasma and (B) plasma spiked with HCTZ 20 ng/mL and I.S.

Spiked concentration (ng mL <sup>-1</sup> )	Determined concentration (ng mL <sup>-1</sup> ) (Mean ± SD) (n=3)	Precision (%) R.S.D.	Accuracy (%) R.E.
5	5.03 ± 0.21	4.17	100.6
20	19.82 ± 0.78	3.94	99.1
30	34.36 ± 2.17	6.32	114.5
80	74.49 ± 1.20	1.61	93.1
100	89.35 ± 2.99	3.35	89.4
200	201.11 ± 9.21	4.58	100.6
300	310.92 ± 6.45	2.07	103.6
400	430.65 ± 10.62	2.47	107.7

**Table 1.** Accuracy and precision of Linearity. SD: Standard Deviation. R.S.D.: Relative Standard Deviation. R.E.: Relative Error.

Spiked conc. (ngmL <sup>-1</sup> )	Intraday (ngmL <sup>-1</sup> ) (n=6)			Interday (ngmL <sup>-1</sup> ) (n=24)		
	Day 1 Mean (R.S.D.)	Day 2 Mean (R.S.D.)	Day 3 Mean (R.S.D.)	Mean	Prec.	Acc. (R.E.)
5	4.37 (10.3 %)	5.24 (10.7 %)	4.72 (9.6 %)	4.773	11.70	95.46
15	16.66 (3.4 %)	14.26 (4.6 %)	13.54 (4.1 %)	14.85	10.18	99.00
190	207.90 (5.4 %)	185.17 (6.2 %)	180.72 (2.8 %)	191.27	7.98	100.67
320	337.24 (3.5 %)	303.07 (7.0 %)	288.41 (3.5 %)	309.57	8.217	96.74

**Table 2.** Precision and accuracy results of validation. R.S.D.: Relative Standard Deviation. R.E.: Relative Error. Prec.: Precision. Acc.: Accuracy. Conc.: Concentration.

concentration of HCTZ was measured in plasma samples from 26 healthy volunteers after single oral dose of 50 mg. The volunteers were submitted a crossover 2x2 study of bioequivalence between a test formulation and reference formulation (Clorana®, SANOFI-SYNTHELABO) with washout of 7 days.

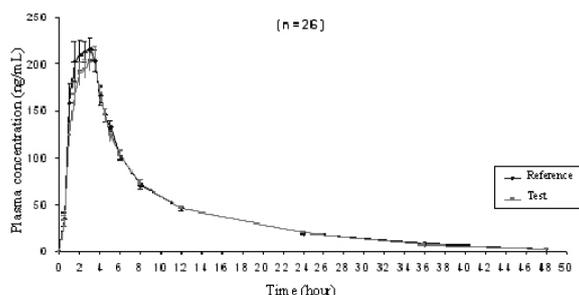
The mean concentration-time profile is shown in Figure 4. Means values of pharma-

cokinetics parameters were: C<sub>max</sub> 244.08 ± 69.01 ng/mL, T<sub>max</sub> 2.65 ± 0.85 h and area under curve (AUC<sub>0-t</sub>) the plasma concentration-time curve 1831.96 ± 459.33 ng/mL.h for test formulation. For the reference formulation the means values for C<sub>max</sub>, T<sub>max</sub> and AUC<sub>0-t</sub> were 254.44 ± 68.38 ng/mL, 2.50 ± 0.95 h and 1882.10 ± 439.54 ng/mL.h, respectively.

The shortest confidence interval (90%) for

Stability Test	Hydrochlorothiazide (n = 4)			
	Nom. Conc. ng/mL	Found Conc. ng/mL	Precision (R.S.D.) (%)	Accuracy (R.E.) (%)
Bench top stability	15	14.39	5.41	-4.06
	320	295.89	2.75	-6.25
Autosampler stability	15	14.43	5.93	-3.80
	320	310.24	3.24	-3.94
Freeze-thaw stability	15	14.21	2.01	-5.27
	320	305.27	2.22	3.69
42-days storage stability	15	15.63	2.68	4.20
	320	341.84	1.54	6.64
Solution stability	15	15.57	3.42	3.80
	320	327.26	2.21	2.27

**Table 3.** Stability data of assay of Hydrochlorothiazide in human plasma. R.S.D.: Relative Standard Deviation. R.E.: Relative Error. Nom.: Nominal. Conc.: Concentration.



**Figure 4.** Mean plasma concentration-time profile of HCTZ after administration of a 50 mg tablet. Data are mean  $\pm$  S.D. for 26 healthy volunteers.

the ratio of  $C_{max}$  means was 87.03 (lower limit) and 105.80 (upper limit). Mean  $C_{max}$  calculated by method of least squares for the reference drug was 254.44 ng/mL and 244.08 ng/mL for the test, resulting in a ratio ( $C_{max\text{test}}/C_{max\text{reference}}$ ) of 95.96 and a test power of 98.11%.

The area under the concentration-time curve from zero time to the time of the last sample collection,  $AUC_{0-t}$  had lower and upper shortest confidence limits (90%) of 90.60 and 103.91, respectively. The  $AUC_{0-t}$  means calculated by method of least squares were 1882.10 ng/mL.h for the reference drug and 1831.96 ng/mL.h for the test drug resulting in a ratio (test/reference) of 97.33% and a test power for  $AUC_{0-t}$  of 99.96%.

## CONCLUSION

We have described a simple, rapid and sensitive LC-MS/MS method for the quantitation of

HCTZ in human plasma, which showed acceptable precision and adequate sensitivity. The major advantages of this method are the simple sample preparation, the short run time (2 min) for high throughput analysis and good sensibility, which are all important characteristics when dealing with large batches of samples. This method has been successfully applied to clinical pharmacokinetics studies of HCTZ in healthy volunteers.

## REFERENCES

1. Bebawy, L.I., S.S. Abbas, L.A. Fattah & H.H. Refaat (2005) *Il Farmaco* **60**: 859-67.
2. Liu, F., Y. Xu, S. Gao, J. Zhang & Q. Guo (2007) *J. Pharm. Biomed. Anal.* **44**: 1187-91.
3. Hillaert, S. & W.V. Bossche (2003) *J. Pharm. Biomed. Anal.* **31**: 329-39.
4. Huang, T., Z. He, B. Yang, L. Shao, X. Zheng & G. Duan (2006) *J. Pharm. Biomed. Anal.* **41**: 644-8.
5. Niopas, I. & A.C. Daftsios (2002) *J. Liq. Chromatogr. Relat. Technol.* **25**: 487-92.
6. Zendelovska, D., T. Stafilov, P. Milosevski (2004) *Biomed. Chromatogr.* **18**: 71-6.
7. Medvedovici, A., C. Mircioiu, V. David, D.S. Miron (2000) *Eur. J. Drug. Metabol. Pharmacokin.* **25**: 91-6.
8. Li, H., Y. Wang, Y. Jiang, Y. Tang, J. Wang, L. Zhao, J. Gu (2007) *J. Chromatogr. B* **852**: 436-42.
9. Ramakrishna, N.V.S., K.N. Vishwottam, S. Manoj, M. Koteswara, S. Wishu & D.P. Varma (2005) *Biomed. Chromatogr.* **19**: 751-60.
10. Takubo, T., H. Okada, M. Ishii, K. Hara, Y. Ishii (2004) *J. Chromatogr. B* **806**: 199-203.