AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

A Validated Reversed-Phase HPLC Method for the Determination of Hydrochlorothiazide in Human Plasma

Syed N. Alvi[1], Muhammad M. Hammami*[1]

1. Clinical Studies and Empirical Ethics Department, King Faisal Specialist Hospital and Research Center, MBC-03, P.O. Box 3354, Riyadh 11211, Kingdom of Saudi Arabia.

ABSTRACT

A simple and precise reversed-phase high performance liquid chromatography (HPLC) method for the determination of hydrochlorothiazide (HCT) in human plasma was developed and validated. Using hydroflumethiazide as an internal standard (IS), separation was achieved on Atlantis dC 18 column. The mobile phase, 10 mM monobasic potassium phosphate and acetonitrile (80:20, v: v), was delivered at a flow rate of 1.2 ml/min. The eluent was monitored by photodiode array detector, with the wavelength set at 272 nm. Plasma samples containing HCT and IS were extracted with methyl tert butyl ether and reconstituted in mobile phase. No interference in blank plasma or of commonly used drugs was observed. The relationship between the concentration of HCT in plasma and peak area ratio of HCT to the IS was linear over the range of 5-300 ng/ml. The intra-day and inter inter-day coefficient of variation and bias were $\leq$ 3.7% and $\leq$ 12.0%, and $\leq$ 10.2% and $\leq$ 7.8%, respectively. Mean extraction recovery of HCT and the IS from plasma samples were 70% and 90%, respectively. The method was applied to assess the stability of HCT under various conditions generally encountered in the clinical laboratory.

Keywords: Hydrochlorothiazide, Hydroflumethiazide, Human plasma, HPLC.

*Corresponding Author Email: muhammad@kfshrc.edu.sa

Received 16 March 2015, Accepted 30 March 2015

Please cite this article as: Hammami MM *et al*., A Validated Reversed-Phase HPLC Method for the Determination of Hydrochlorothiazide in Human Plasma. American Journal of PharmTech Research 2015.

INTRODUCTION

Hydrochlorothiazide (HCT, CAS: 58-93-5, 6-Chloro-3,4-dihydro-2H-1,2,4-benzothiazide-7-sulfonamide 1,1-dioxide) is a potent thazide diuretic widely used in treatment of hypertension [1, 2]. Its oral bioavailability is around 70%, with a mean peak plasma concentration of 77 ± 26.5 ng/ml at about 1.0 - 2.5 hours after the ingestion of one 12.5 mg therapeutic dosage [3, 4]. Figure 1 depicts the structure of HCT and the internal standard (hydroflumethiazide, IS) used in the study. Several analytical methods have been reported for the determination of HCT in pharmaceutical formulations, individually [5,] or in combination with others antihypertensive drugs such as irbesartan, benazepril hydrochloride, cilazapril, losartan and/or amlodipine [6-10]. HCT level in human plasma or serum has been mainly determined by high performance liquid chromatography (HPLC) with ultra violet detection (UV) [11-14] or liquid chromatography mass spectrometry (LCMS) [15-17]. However, some of these methods were not sufficiently sensitive and/or not fully validated[11-14], and some used expensive equipment [15-17]. This paper describes a simple HPLC method which enables the determination of HCT level in human plasma with great accuracy even at a low concentration. The method was fully validated and successfully applied in stability studies.

MATERIALS AND METHOD

Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 (Waters Associates Inc, Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. A reversed-phase column Atlantis dC18 (4.6 x 150 mm, 5-µm) and a guard pak pre-column module with a Nova pak C18, 4-µm insert were used for the separation. The data were collected with a Pentium IV computer using Empower Chromatography Manager Software [18].

Chemical and reagents

All reagents were of analytical-reagent grade unless stated otherwise. HCT and IS were purchased from Sigma-Aldrich Chemie, Steinheim, Germany. Acetonitrile (HPLC grade) and monobasic potassium phosphate were purchased from Fisher Scientific, Fairlawn, NJ, USA. Water for HPLC analysis was generated by reverse-osmosis and further purified by passing through a Synergy Purification System (Millipore Co., Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia.

Chromatographic conditions

The mobile phase composed of 10 mM monobasic potassium phosphate (pH 3.5, adjusted with phosphoric acid), and acetonitrile (80:20, v:v). Before delivering into the system, the mobile phase was filtered through 0.45 µm polyetersulfone membrane and sonicated under vacuum for 5 minutes. The analysis was carried out under isocratic conditions using a flow rate of 1.2 ml/min at 23 ºC and a run time of 10 minutes. Chromatograms were recorded at 272 nm using a photodiode array detector.

Preparation of standard and quality control samples

Stock solution of HCT and IS (0.1 mg/ml) were prepared in mobile phase. They were diluted with blank human plasma and mobile phase to produce working solutions of 0.5 µg/ml and 1.0 µg/ml, respectively. Nine calibration standards in the range of 5 - 300 ng/ml were prepared in human plasma. Four quality control (QC) samples (5, 15, 150, 270 ng/ml) were prepared in human plasma. The solutions were vortexed for one minute, then 1.0 ml aliquots were transferred into teflon-lined, screw capped, borosilicate, 13 X100 mm glass culture tubes, and stored at -20 ºC until used.

Sample preparation

Aliquots of 1.0 ml of calibration standards or QC samples were allowed to equilibrate to room temperature. To each tube, 200 µl of the IS working solution (1 µg/ml in mobile phase) were added and the mixture was vortexed for 10 seconds. After the addition of 5 ml of methyl tert butyl ether, samples were vortexed again for 1 min and then centrifuged for 15 min at 4200 rpm at room temperature. The organic layer was carefully collected into a clean tube and evaporated to dryness under a gentle steam of nitrogen on multiplace heating block at temperature not exceeding 40 ºC, and the residue was reconstituted in 150 µl mobile phase then centrifuged at 8400 rpm for 5 min at room temperature. The supernatant was transferred into an auto-sampler vial and 100 µl were injected into the HPLC system.

Stability studies

Three QC samples (5, 15, and 270 ng/ml) were used for HCT stability studies: Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at –20 °C for eight weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed, reconstituted, and stored at room temperature for 24 hours or 48 hours at –20 °C

before analysis (autosampler stability). Finally, fifteen aliquots of each QC sample were stored at –20 °C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to –20 °C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [19]. The validation parameter included, specificity, linearity, accuracy, precision, recovery, and stability.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Under the optimal experimental conditions, a mobile phase composed of 10 mM monobasic potassium phosphate (pH=3.5 ±0.02, adjusted with phosphoric acid) and acetonitrile (80:20, v:v) and a flow rate of 1.2 ml/min, HCT, IS, and components of plasma exhibited a well defined separation within 10 minutes run. The retention times of HCT and IS were around 4.8 and 8.8 minutes, respectively.

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in plasma samples include endogenous components, metabolites, and decomposition products. We screened six batches of blank plasma and eight frequently used medications (aspirin, acetaminophen, ranitidine, nicotinic acid, ascorbic acid, caffeine, ibuprofen, and diclofenac) for potential interference. No interference was found in plasma and none of the drugs co-eluted with HCT or the IS. Figure 2 depicts a representative chromatogram of a drug free human plasma used in preparation of standard and quality control samples.

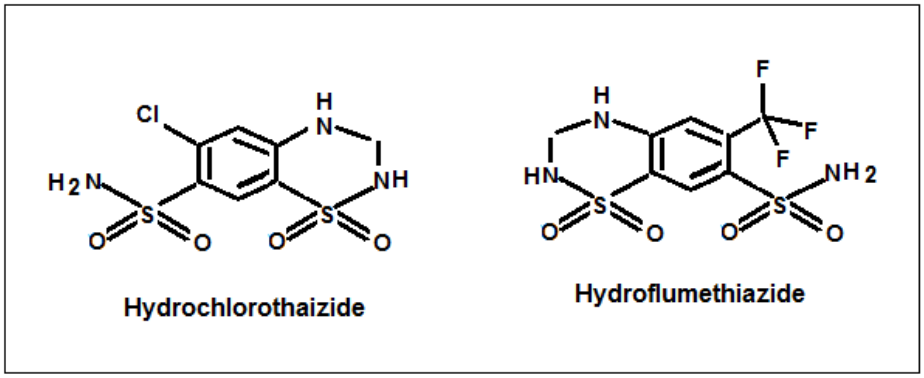

Figure 1: Chemical structures of hydrochlorothiazide and hydroflumethiazide (IS).

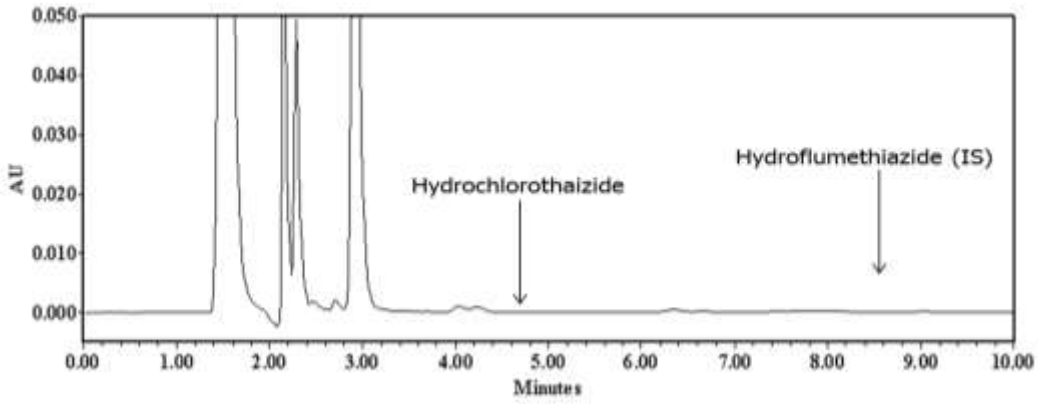

Figure 2: Representative chromatogram of drug-free human plasma. The arrows indicate the retention times of hydrochlorothiazide (4.8 min.) and hydroflumethiazide (8.8 min.)

Limit of quantification (LOQ) and linearity

The LOQ was defined as the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation and bias ≤ 20%). The LOQ of HCT in human plasma was 5.0 ng/ml and gave a response of analyte to blank > 5. The lowest detection limit (analyte to blank response ≥ 3) was 2.5 ng/ml. Linearity of HCT was evaluated by analyzing ten curves of nine concentrations over the range (5-300 ng/ml) prepared in human plasma. Figure 3 represents an overlay of chromatograms of extracts of 1.0 ml human plasma spiked with the IS and one of nine concentrations of HCT. The peak area ratios were subjected to regression analysis. The mean regression equation was y = 0.0052 x – 0.172. The suitability of the calibration curves was confirmed by back-calculating the concentration of HCT in human plasma from the calibration curves (Table 1). All calculated concentrations were well within the acceptable limits.

Table 1: Back-calculated hydrochlorothiazide concentrations from ten calibration curves

Nominal level (ng/ml)	Calculated level (ng/ml)		CV (%)	Bias (%)
	Mean	SD		
5	4.7	0.50	10.7	-6.2
10	9.6	0.89	9.2	-3.6
20	18.1	1.13	6.2	-9.3
30	28.5	1.74	6.1	-5.0
60	56.0	2.94	5.3	-6.7
90	83.7	5.67	6.8	-7.1
120	110.5	2.93	2.6	-7.9
240	226.5	6.48	2.9	-5.6
300	308.6	6.16	2.0	2.9

SD, standard deviation CV, standard deviation divided by mean measured concentration x100.

Bias, measured level - nominal level divided by nominal level x 100.

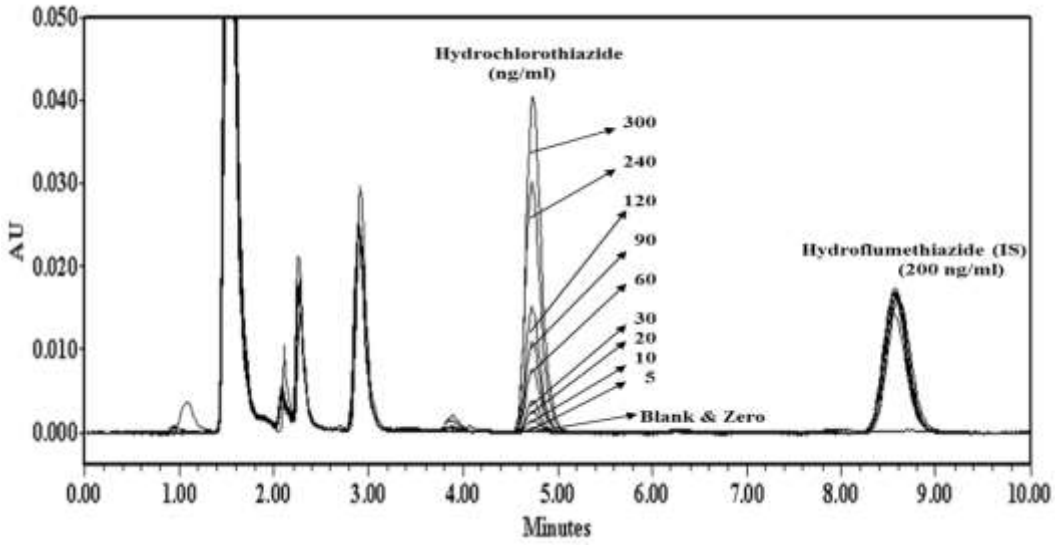

Figure 3: Overlay of chromatograms of extracts of 1.0 ml human plasma spiked with the internal standard (IS) and one of ten concentrations of HCT (0, 5, 10, 20, 30, 60, 90, 120, 240, and 300 ng/ml).

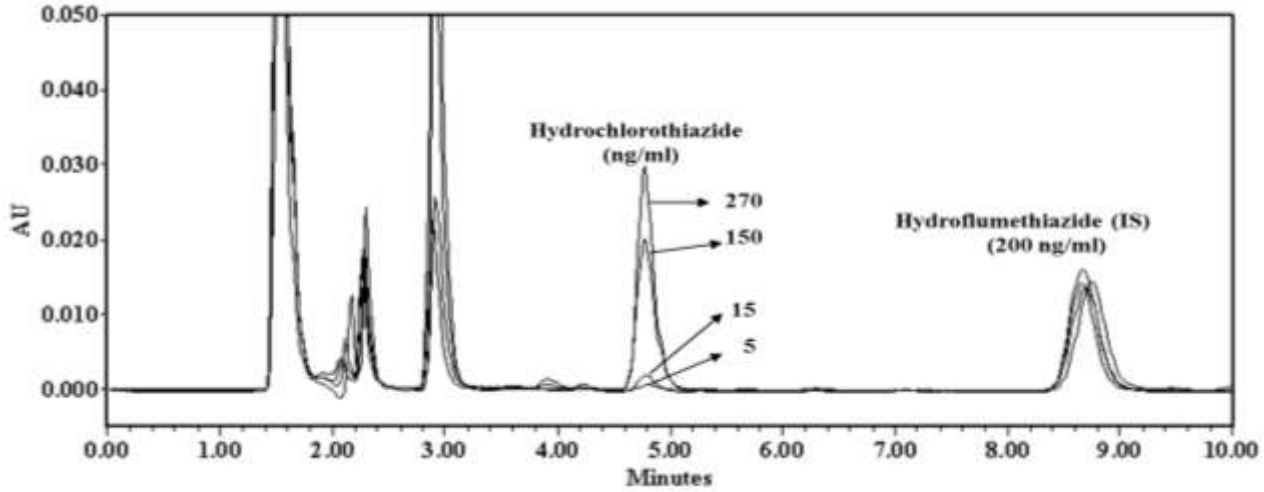

Figure 4: Representative overlay chromatogram of quality control samples (concentrations: 5, 15, 150, and 270 ng/ml) spiked with the internal standard (IS).

Accuracy and Precision

According to predetermined criteria [19], accuracy and precision were determined using four QC concentrations (5, 15, 150, and 270 ng/ml). The inter-day precision and accuracy of the assay were determined over three different days. The intra-day (n=10) and inter-day (n=20) precision were ≤ 3.7 % and ≤ 10.2 %, respectively. The intra-day and inter-day bias were in the range of -12.0 to 3.8 % and -7.8 to 3.5 %, respectively. The results are summarized in Table 2. The results indicate that the method was reliable within the studied concentration range.

Table 2: Intra and inter-day bias and precision of hydrochlorothiazide assay

	Intra-run (n=10)				Inter-run (n=20)			
Nominal level (ng/ml)	**Measured level (ng/ml)**		**CV (%)**	**Bias (%)**	**Measured level (ng/ml)**		**CV (%)**	**Bias (%)**
	Mean	**SD**			**Mean**	**SD**		
5	4.4	0.16	3.7	-12.0	4.7	0.48	10.2	-6.0
15	14.9	0.34	2.3	-0.7	15.3	0.93	6.1	2.0
150	155.7	2.96	1.9	3.8	155.2	2.50	1.6	3.5
270	248.8	7.50	3.0	-7.9	249.0	6.65	2.7	-7.8

SD, standard deviation CV, standard deviation divided by mean measured concentration x100.

Bias, measured level - nominal level divided by nominal level x 100.

Recovery

The absolute recovery of HCT was assessed by direct comparison of absolute peak areas from plasma vs. mobile phase samples, using five replicates for each of four QC concentrations (5, 15, 150 and 270 ng/ml). Similarly, the recovery of the IS was determined by comparing the peak areas of the IS in 5 aliquots of human plasma spiked with 200 ng/ml IS with the peak areas of equivalent samples prepared in mobile phase. The results are presented in Table 3. Mean recovery for HCT and IS were 70% and 90%, respectively.

Table 3: Recovery of hydrochlorothiazide and the internal standard from 1.0 ml of human plasma.

Concentration (ng/ml)	**Human plasma***	**Mobile phase***	**Recovery (%)**
Hydrochlorothiazide			
5	5797 (597)	8513 (209)	68
15	18185 (550)	26433 (283)	69
150	198505 (2462)	284060 (563)	70
270	369746 (11990)	519050 (1010)	71
Internal Standard			
200	303204 (10221)	337216 (932)	90

* Data represent mean peak area (SD), n = 5.

Stability

Stability of analyte is an important pre-analytical variable. It is necessary to perform stability studies to determine the range of appropriate conditions and time of storage. HCT and IS stability in processed and unprocessed plasma samples was investigated. HCT in processed samples (5, 15 and 270 ng/ml) was found to be stable for 24 hours at room temperature (≥ 97%) and 48 hours at -20 ºC (≥ 96%). HCT in unprocessed plasma samples was stable for at least 24 hours at room temperature (≥ 91%), eight weeks at -20 ºC (≥ 98%) and after three freeze-and thaw cycles (≥ 102%). Data are summarized in Table 4. HCT and IS in stock solutions (0.1 mg/ml in mobile

phase) were found to be stable for 24 hours at room temperature (101%) and at least eight weeks at -20 ºC (100%). Further, no significant change in chromatographic behavior of HCT or the IS was observed under any of the above conditions.

Table 4: Stability of hydrochlorothiazide under various clinical laboratory conditions

Plasma Samples: Stability (%)							
Nominal level (ng/ml)	**Unprocessed**		**Processed**		**Freeze –Thaw Cycle**		
	(24 hrs RT)	**(8 wks, -20 °C)**	**(24 hrs,RT)**	**(48 hrs, -20 °C)**	**1**	**2**	**3**
5	91	112	97	103	110	103	104
15	101	98	100	98	104	102	104
270	98	98	97	96	103	105	105

CONCLUSION

In summary, the described HPLC method for the determination of HCT in human plasma is rapid, simple, precise, and accurate. The assay was validated to meet the requirements of pharmacokinetic or bioequivalence studies. It was applied to monitor the stability of HCT under various clinical laboratory conditions.

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