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ORIGINAL RESEARCH

Development of solid-phase extraction method and its application for determination of hydrochlorothiazide in human plasma using HPLC

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ABSTRACT: A high-performance liquid chromatographic method was developed, validated and applied for the determination of hydrochlorothiazide in human plasma. The effects of mobile phase composition, buffer concentration, mobile phase pH and concentration of organic modifiers on retention of hydrochlorothiazide and internal standard were investigated. The method involves 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 recovery, selectivity, linearity, precision and accuracy of the method were evaluated from spiked human plasma samples. Limit of quantification was 10 ng mL^{-1}. The method has been implemented to monitor hydrochlorothiazide levels in patient samples. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: thiazide diuretics, reversed-phase HPLC; UV detection; method validation

INTRODUCTION

Hydrochlorothiazide and the other thiazide diuretics are used in the treatment of oedema associated with heart failure and with renal and hepatic disorders. They are also used in hypertension, either alone or together with other antihypertensive agents, such as angiotensin-converting enzyme (ACE) inhibitors and beta blockers (Martindale, 1996).

Several high-performance liquid chromatography methods have been developed for measuring hydrochlorothiazide concentration in pharmaceutical preparations (*European Pharmacopoeia*, 2002; Daniels and Vanderwielen, 1981; Stewart and Clark, 1986; Kartal and Erk, 1999; Zecevic *et al.*, 2000; Hertzog *et al.*, 2002; Jonczyk and Nowakowska, 2001).

There have been numerous publications describing the determination of hydrochlorothiazide concentrations in plasma or urine by high-performance liquid chromatography (HPLC) with ultraviolet or electrochemical detection after liquid–liquid extraction with various organic solvents. (Alton *et al.*, 1986; Shiu *et al.*, 1986; Medvedovici *et al.*, 2000; de Vries and Voss, 1993; Koopmans *et al.*, 1984; Barbhaiya *et al.*, 1981; Richter *et al.*, 1996). However, the disadvantage of these methods employing liquid–liquid extraction of hydrochlorothiazide from biological fluids is that they involve several steps yielding poor separation from the plasma and urine endogenous interferences and gave highly variable and relatively low recoveries. Farthing *et al.* (1998) reported liquid chromatographic method utilizing narrowbore chromatography for determination of hydrochlorothiazide in human urine.

Fett *et al.* (1991) have performed the determination of hydrochlorothiazide in urine using micellar liquid chromatography by direct injection of the sample into the chromatographic system. On the other hand, Bonet *et al.* (1992) proposed liquid chromatographic determination of diuretics in urine through direct injection without any sample pretreatment using micellar liquid chromatography. However, they reported that the urine matrix overlapped some of the investigated diuretics including hydrochlorothiazide.

Kuo *et al.* (1990) proposed an automated high-performance liquid chromatographic method for hydrochlorothiazide in plasma using column-switching technique. This method involves direct injection of plasma to the extraction column for sample clean-up followed by switching onto the analytical column. The only disadvantage of this method is that takes great reagent consumption and HPLC system with two pumps.

No currently available methods were found for the determination of hydrochlorothiazide in human

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plasma using solid-phase extraction. The aim of this work is to present solid-phase extraction method for the elimination of plasma endogenous interference's on the determination of hydrochlorothiazide in human plasma. Namely, to continue our work on development of solid-phase extraction methods for separation of drug from the plasma endogenous interferences (Zendelovska *et al.*, 2002a,b), in this paper we propose a new method for determination of hydrochlorothiazide in plasma samples using solid-phase extraction technique on RP-select B cartridges. In order to fulfil the aim, method was first developed for the separation of and determination of hydrochlorothiazide concentrations using internal standard method by optimizing the experimental parameters and determining linearity for the investigated drug. The method was then validated by evaluating recovery, selectivity, linearity, precision and accuracy. Finally, the method was used for determination of hydrochlorothiazide in patient plasma samples.

EXPERIMENTAL

Reagents and chemicals

Hydrochlorothiazide and internal standard (IS) caffeine, were kindly supplied by Changzhou Pharmaceutical Factory (China) and Sigma (Germany), respectively. Acetonitrile and methanol were purchased from Across Organics (Belgium); *o*-phosphoric acid, potassium dihydrogen phosphate and triethylamine were obtained from Merck (Germany). All reagents used were of analytical grade except acetonitrile and methanol, which were HPLC grade. Cartridges for solid-phase extraction were purchased from Merck (Germany).

Chromatographic system

The HPLC analysis was carried out on a Perkin-Elmer liquid chromatography system (USA) consisted of a pump (LC series 200), an autosampler (LC ISS Series 200), a diode array detector (LC 235 C) and a column oven (model 101). The chromatographic system was controlled by software package Turbochrom Version 4.1. plus and UV-spectrometric data were produced by program TurboScan Version 2.0. The analytical column was Hibar Lichrospher 100 RP-8, 250 × 4 mm i.d., 5 μm particle size with matched guard column (Merck, Germany). The mobile phase consisted of 0.025 mol L^{-1} phosphate buffer (pH 5 adjusted with very small amount of triethylamine)–acetonitrile (85:15, v/v). The mobile phase was filtered, degassed with helium and delivered at a flow-rate of 1.2 mL min^{-1}. All experiments were carried out at an ambient temperature of approximately 25°C.

Preparation of standard solutions and plasma samples

Stock solutions of hydrochlorothiazide and caffeine were prepared at concentration 1000 μg mL^{-1}. Stock solution of hydrochlorothiazide was prepared by dissolving 0.05 g in 7 mL of 0.1 mol L^{-1} NaOH and 43 mL of water. Working stock solution of caffeine was prepared in water. These solutions were stored at 4°C and no change in stability over the period of 1 month was observed. The working solutions were prepared by diluting appropriate portions of these solutions with distilled water.

Human plasma was prepared from heparinized whole blood samples. Blood samples were collected from healthy volunteers and stored at –20°C. After thawing, samples were spiked daily with working solutions of hydrochlorothiazide and internal standard.

Sample preparation

A solid-phase extraction vacuum manifold (Merck) was used for sample preparation. Satisfactory values for recovery of hydrochlorothiazide and IS were obtained with a single extraction with RP-select B solid phase cartridge (200 mg) for isolation of the drugs from plasma samples. Before analyses plasma samples (1 mL) spiked with 100 μL of IS (10 μg mL^{-1}) were buffered with 1 mL of 0.1 mol L^{-1} KH_2PO_4 solution (pH 3 adjusted with *o*-phosphoric acid). The cartridge was conditioned sequentially by 2 mL of methanol and 2 mL of water. The buffered plasma sample was introduced into the cartridge under vacuum at 5 psi. Water (3 mL) was used to rinse the cartridge. The analytes were eluted with 2 mL of methanol. The eluate was evaporated to dryness under N_2 for about 20 min at 40°C. After reconstitution of the residue with 200 μL of mobile phase, the sample was filtered using filter with pore size of 0.45 μm and a 100 μL volume was injected into the HPLC system.

Extraction recovery

Recovery studies were performed by analysing plasma samples spiked with hydrochlorothiazide at three concentration levels. Three replicate samples for each concentration were extracted and chromatographed. The extraction recoveries were calculated by comparing the peak height of hydrochlorothiazide and internal standard obtained for low, medium and high level quality control samples ($n = 3$ for each level for hydrochlorothiazide, $n = 9$ for internal standard) and those resulting from the direct injection ($n = 3$, working solutions) of the theoretical amount of either hydrochlorothiazide or internal standard (=100% recovery).

Assay validation for hydrochlorothiazide

Linearity. Six calibrators of hydrochlorothiazide were prepared by making serial dilutions from stock solution and spiking them into drug-free human plasma. The standard samples were prepared according to the procedures as unknown samples. The calibration curve was constructed by analysing a series of plasma calibration samples spiked with hydrochlorothiazide to obtain concentrations ranging from 10.0 to 900.0 ng mL^{-1}. The chromatograms were evaluated on the basis of hydrochlorothiazide/IS ratios of the peak heights. The regression equation was calculated by the least-squares method.

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Precision, accuracy and ruggednesss. Intra- and inter-day precision was determined in three different days by analysing spiked plasma samples from each concentration used for construction of calibration curves. The mean, standard deviations and RSD of the intra- and inter-day experiments were calculated.

Intra- and inter-day accuracy was determined by measuring plasma quality control samples at low, middle and high concentration levels of hydrochlorothiazide. Relative error of mean predicted concentration compared with nominal concentration was determined.

Ruggedness was tested on the second HPLC column of the same type by determining linearity, precision and accuracy. Linearity was performed at six concentration points for hydrochlorothiazide in human plasma in concentration range from 10.0 to 900.0 ng mL^{-1}. Intra-day precision and accuracy were determined by measuring two series of plasma quality control samples.

Stability evaluation. The stability of hydrochlorothiazide in plasma was investigated using spiked samples at two different concentration levels prepared in duplicate. Spiked samples were analysed after different storage conditions: immediately, after staying in an autosampler for 2, 12 and 24 h, after one and two freeze/thaw cycles and after one month stored at –20°C. Stability was determined by comparing the nominal concentration of hydrochlorothiazide in samples analysed immediately and the test samples.

RESULTS AND DISCUSSION

Method development

A series of studies were conducted in our laboratory in order to develop a convenient and easy-to-use method for quantitative analysis of hydrochlorothiazide in human plasma. Several HPLC method variables with respect to their effect on the separation of hydrochlorothiazide and internal standard from the matrix were investigated. In our extensive preliminary experiments a set of column packing including C_8, C_{18} and RP-select B with different lengths and particle sizes was tested. The final choice of the stationary phase giving satisfying peak shape, resolution and run time was a Hibar Lichrospher 100 RP-8 (250 × 4 mm i.d.; 5 μm particle size). Also, a series of aqueous mobile phases containing buffer solutions with different pH values in combination with different modifiers including acetonitrile, methanol and triethylamine with different volume fractions were tested. The elution was monitored in the whole UV region and the wavelength of 230 nm exhibited the best detection.

The amount of organic modifier present in the mobile phase influences analytes that are retained predominantly by adsorption onto the stationary phase. Figure 1 shows the results that were obtained over an acetonitrile range of 10–25% in the mobile phase 0.025 mol L^{-1} KH_2PO_4, pH 5 adjusted with very small amount of triethylamine. This data was used to determine an optimal amount of organic modifier that should be used for the separation of hydrochlorothiazide and IS. The best results (good separation between two peaks, short time of analysis) can be obtained when the percentage of acetonitrile in the mobile phase is 15%.

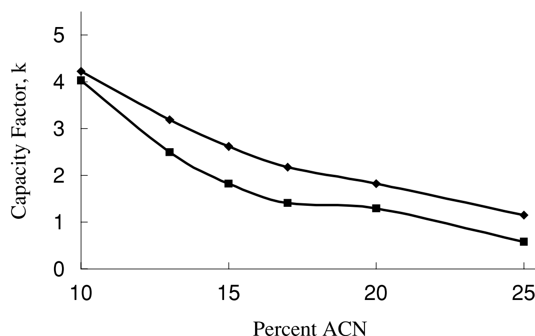

Figure 1. The effect of the organic modifier concentration on analytes retention (◆, hydrochlorothiazide; ■, caffeine).

Additional studies were also done to check the effect that mobile phase pH and buffer concentration had on analytes retention and resolution. Results from this investigation showed that there was no significant change in retention and resolution of investigated drugs when the concentration of buffer was increased from 0.01 to 0.1 mol L^{-1}, and the concentration of 0.025 mol L^{-1} was chosen to minimize the equilibration time of the column after each analytical run. The effect of pH on the retention and separation was observed over the range 3.0–7.0 using phosphate as buffer salt. The retention and resolution of hydrochlorothiazide and internal standard were largely unaffected by changes in pH. An intermediate value 5.0 was chosen so as to avoid pH extremes and thus prolong column life.

From these data it was determined that mobile phase consisted of 0.025 mol L^{-1} KH_2PO_4, pH 5 and 15% acetonitrile would provide good retention for hydrochlorothiazide and caffeine as well as an acceptable run time of less than 10 min for the separation.

Typical chromatograms of standard solutions of hydrochlorothiazide and caffeine produced by the developed HPLC method are shown in Fig. 2(c). The retention times of the internal standard and hydrochlorothiazide were 5.4 and 6.8 min, respectively.

In addition, different cartridges for solid phase extraction (C_{18} and RP-select B) were tested in order to obtain satisfactory values for recovery of hydrochlorothiazide and caffeine. Results from this investigation show that the satisfactory values for recovery of hydrochlorothiazide and IS were obtained when plasma samples were extracted with RP-select B cartridges. When solid-phase extraction with RP-select B cartridge

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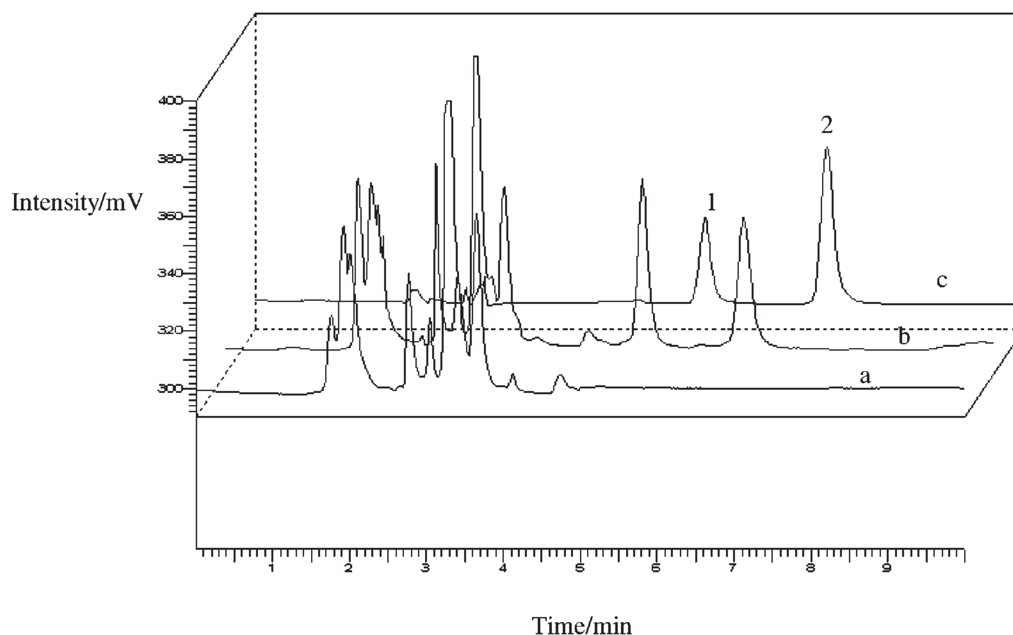

Figure 2. Chromatograms of standard solutions (c) of hydrochlorothiazide and IS, caffeine (2.5 μg mL^{-1}); 1-internal standard, 2-hydrochlorothiazide, blank (a) and spiked plasma (b) samples containing 400 ng mL^{-1} of hydrochlorothiazide.

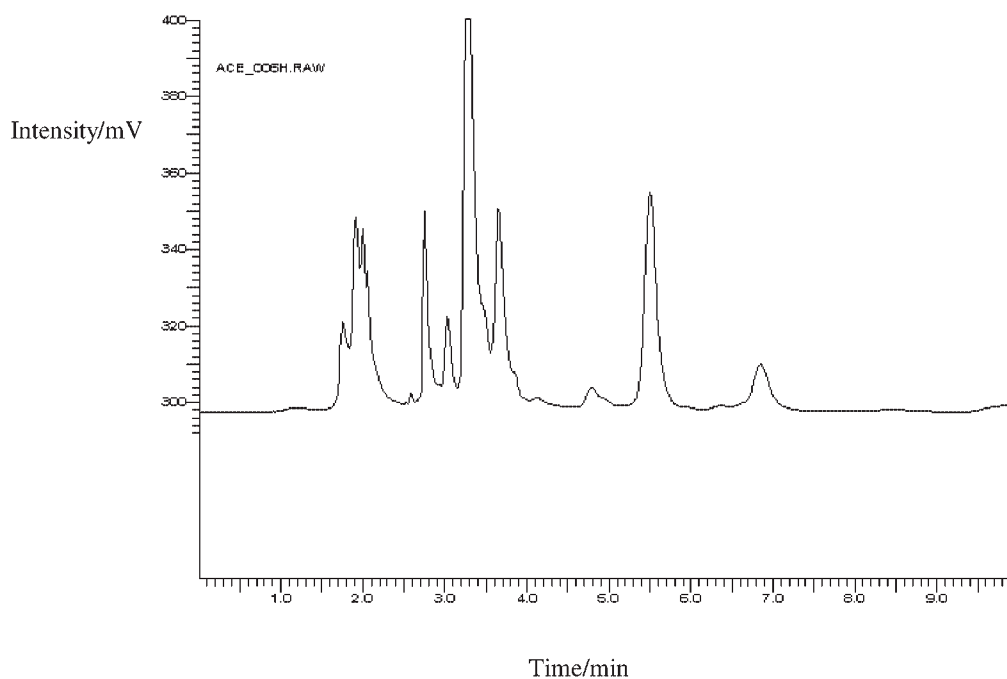

Figure 3. Chromatogram of patient plasma sample after oral administration of 25 mg of hydrochlorothiazide.

was used for sample preparation, values for recovery for hydrochlorothiazide and IS ranged from 97.2 to 104.1%. On other hand, when solid-phase extraction was performed on C_{18} cartridges, values for recovery ranged from 66.7 to 69.4% for both drugs.

Typical chromatograms of the blank human plasma (a) and plasma sample spiked with hydrochlorothiazide and internal standard (b) are shown in Fig. 2. No interfering peaks were found at the retention times of hydrochlorothiazide and internal standard.

The developed HPLC method was used for analysis of patient plasma samples after oral administration of hydrochlorothiazide. Typical chromatograms of plasma samples of patients prepared according procedures for sample preparation after a single 25 mg oral dose of hydrochlorothiazide are shown in Fig. 3.

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Table 1. Intra- and inter-day precision data

Hydrochlorothiazide nominal concentration (ng mL^{-1})	Intra-day		Inter-day	
	Mean (n = 3) observed concentration (ng mL^{-1})	Relative standard deviation (%)	Mean (n = 6) observed concentration (ng mL^{-1})	Relative standard deviation (%)
10.0	9.31	6.02	9.43	6.99
50.0	48.02	3.87	50.47	6.12
100.0	106.4	2.81	106.7	4.18
250.0	257.4	2.57	254.8	1.90
400.0	390.6	0.18	390.0	0.60
900.0	901.3	0.79	902.1	1.02

Table 2. Intra- and inter-day accuracy data

Hydrochlorothiazide nominal concentration (ng mL^{-1})	Intra-day		Inter-day	
	Mean (n = 3) observed concentration (ng mL^{-1})	Relative error (%)	Mean (n = 6) observed concentration (ng mL^{-1})	Relative error (%)
25.0	25.02	0.09	24.11	–3.56
150.0	147.9	–1.36	150.1	0.09
300.0	303.2	1.08	295.8	–1.39

Method validation

Linear detector response for the peak-height ratios of hydrochlorothiazide to internal standard was observed in concentration range between 10.0 and 900.0 ng mL^{-1} with a correlation coefficient of 0.9997. The respective regression equation for hydrochlorothiazide obtained after preparation of samples using solid-phase extraction was: $y = 0.002 \cdot \gamma + 0.0071$.

Under the experimental conditions used, the lower limit of detection was 3 ng mL^{-1} at a signal-to-noise ratio of 3. The limit of quantification was defined as the lowest amount detectable with a precision of less than 15% (n = 5) and an accuracy of ±15% (n = 5). The limit of quantification was found to be 10 ng mL^{-1}.

The results of the method validation study are presented in Tables 1 and 2. The intra- and inter-day variations of the method throughout the linear range of concentrations are shown in Table 1. The intra- and inter-day reproducibilities expressed as relative standard deviation (RSD) were found to be 0.18–6.99%, indicating good precision of the proposed method. An indication of accuracy was based on the calculation of the relative error of the mean observed concentration as compared with the nominal concentration. Accuracy data are presented in Table 2. Relative errors at all three concentrations studied are less than 3.56% and it is obvious that the method is remarkably accurate, ensuring reliable results.

Ruggedness was tested on the second HPLC column of the same type by determining linearity, precision and accuracy. Linearity was performed at six concentration points for hydrochlorothiazide in human plasma in concentration range from 10.0 to 900.0 ng mL^{-1}. The regression equation was: $y = 0.0019 \cdot \gamma + 0.0104$. Also, the correlation coefficient was 0.9997. Relative standard deviations at all three concentrations studied for hydrochlorothiazide were less than 1.78%. The relative errors ranged from 0.04 to 4.46% of the nominal concentrations of investigated drug. As can be seen, the results of this assessment are very similar to those obtained by previous investigation on the first HPLC column. That means that this HPLC method for determination of hydrochlorothiazide in spiked human plasma samples is rugged.

The results from the stability studies show that relative errors at two different concentrations studied are less than 4.32% and it is obvious that hydrochlorothiazide added to plasma are stable in the different storage conditions.

CONCLUSION

The proposed HPLC method employing solid-phase extraction for sample preparation is simple and convenient for the determination of hydrochlorothiazide in plasma samples. The typical assay time is about 10 min. The proposed method is simply, rapid and provides efficient clean up of the complex biological matrix and high recovery of hydrochlorothiazide and internal standard. The validation data demonstrate good precision and accuracy, which proves the reliability of the proposed method. Finally, the method has been implemented to monitor hydrochlorothiazide levels in clinical samples.

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