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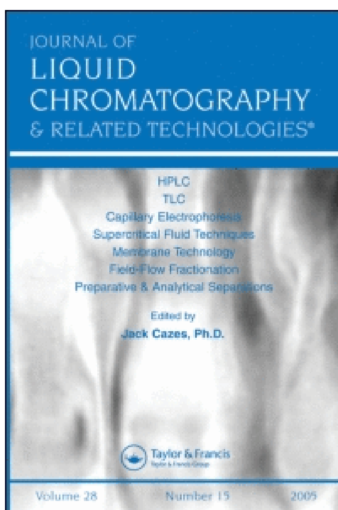

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A VALIDATED HPLC METHOD FOR THE DETERMINATION OF HYDRO-CHLOROTHIAZIDE IN HUMAN PLASMA AND ITS APPLICATION IN PHARMACOKINETIC STUDIES

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ABSTRACT

A simple, specific, sensitive, precise, and accurate high performance liquid chromatography procedure was developed and validated for the analysis of hydrochlorothiazide in human plasma. After addition of hydroflumethiazide as internal standard, the analytes were isolated from human plasma by double liquid-liquid extraction and separated on a reversed phase column with acetonitrile/water 0 : 80, v/v as the eluent. Peaks were monitored at 271 nm. Peak heights were measured and hydrochlorothiazide was quantified using calibration curves.

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The method was found to be linear in the 5 to 80 ng/mL concentration range ($r > 0.999$). The limit of quantitation was found to be 5 ng/mL for 1 mL plasma samples. The inter-assay precision, expressed as the coefficients of variation (CV%), ranged from 2.7 to 9.4% and the assay inaccuracy was within 2.2%.

The method was applied to estimate the pharmacokinetics of hydrochlorothiazide after administration of a single oral dose containing 20 mg enalapril and 12.5 mg hydrochlorothiazide to 24 healthy volunteers.

INTRODUCTION

Hydrochlorothiazide (6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfon-amide 1,1-dioxide) is a potent thiazide diuretic that enhances natriuresis, which leads to a reduction in plasma volume and cardiac output. Therefore, it is used widely alone, or in combination with, other antihypertensive drugs for the treatment of hypertension and congestive cardiac failure (1,2). The most common adverse effects are hypokalaemia, hyperuricaemia, decreased glucose tolerance, hyperlipidaemia, impotence, and lethargy. Hydrochlorothiazide is readily absorbed from the gastrointestinal tract and its absolute bioavailability following oral administration is approximately 70%. It has a volume of distribution (V) about 210 liters and its total clearance (CL) is approximately 22 L/h. Peak plasma concentrations (C_{max}) are achieved in about 2 hours after oral administration and its elimination half-life ($t_{1/2}$) is approximately 10 hours. The bioavailable dose fraction is excreted almost completely in the urine (2–4).

Several HPLC methods have been reported for the determination of hydrochlorothiazide in plasma and urine (5–10). However, some of these methods were not sufficiently specific and sensitive, some were not validated, and some were expensive and not directly applicable for the quantitation of hydrochlorothiazide in human plasma.

This report describes the development and validation of an analytical procedure for the determination of hydrochlorothiazide in human plasma. The method was proved to be suitable for pharmacokinetic studies that required high selectivity and sensitivity.

EXPERIMENTAL

Chemicals

Hydrochlorothiazide was obtained from Elpen (Athens, Greece) and the internal standard hydroflumethiazide was purchased from Sigma (St. Louis, MO,

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USA). Acetonitrile and *n*-hexane, both of HPLC grade, were obtained from J.T. Baker (Deventer, Netherlands). *Tert*-butylmethyl ether, of HPLC grade, was purchased from Riedel-deHaen (Seelze, Germany). Water was Milli-Q grade and all other chemicals and solvents used were of analytical grade.

Instrumentation and Chromatographic Conditions

The HPLC apparatus consisted of a Shimadzu LC-600 delivery pump (Columbia, MD, USA), a Hitachi 655A-40 autosampler (Tokyo, Japan), an ISCO V-4 variable wavelength UV-Vis detector (Lincoln, NE, USA), and a Hewlett-Packard HP3396A integrator (Avondale, PA, USA). Chromatography was conducted using a Zorbax reversed-phase C_{18} analytical column (250×4.6 mm I.D.), particle size 5 μm (Rigas Labs, Thessaloniki, Greece). The mobile phase was acetonitrile/Milli-Q grade water (20 : 80, v/v) and was filtered through a 0.45 μm pore size nylon filter and degassed by ultrasonic treatment before use. The HPLC system was operated isocratically at a flow rate of 1 mL/min at ambient temperature and peaks were detected at 271 nm. Under these conditions hydrochlorothiazide and hydroflumethiazide were eluted in 5.8 and 9.0 min, respectively.

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Preparation of Standards

Stock solutions of hydrochlorothiazide and the internal standard hydroflumethiazide were prepared daily by dissolving appropriate amounts of the compounds in acetonitrile to give concentrations of 200 μg/mL and 500 μg/mL for each compound, respectively. Appropriate dilutions of the stock solution of hydrochlorothiazide were made with 25% acetonitrile/water to prepare working solutions containing 200, 400, 800, 1600, and 3200 ng/mL of hydrochlorothiazide. Twenty-five μL of these working solutions were used to spike 1.0 mL of plasma samples to prepare the calibration curves containing 5, 10, 20, 40, and 80 ng/mL hydrochlorothiazide. The stock solution of the internal standard was further diluted with 25% acetonitrile/water to prepare the working internal standard solution containing 10 μg/mL hydroflumethiazide. Ten μL of this solution was added to each plasma sample for the analysis.

Volumes of 50 mL of human plasma were spiked with appropriate volumes of stock solution to obtain quality control samples containing 12, 23, and 45 ng/mL hydrochlorothiazide, respectively. These samples were divided into aliquots of about 3 mL into one-dram vials capped tightly, and placed at −20°C pending analysis.

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Sample Preparation

A 1.0-mL aliquot of human plasma, 25 µL of working hydrochlorothiazide solution, 10 µL of working internal standard solution, and 1 mL of 0.1 M sodium bicarbonate solution were added in a 13 × 100 mm glass test tube and mixed well. To control blanks and to quality control standards, 35 and 10 µL of 25% acetonitrile/water were added, respectively. The samples were shaken with 5 mL of *tert*-butylmethyl ether for 10 minutes and centrifuged at 3000 *g* for 10 minutes. The organic phase was transferred into a 13 × 100 mm glass test tube and evaporated to dryness at 45°C with the aid of a gentle stream of filtered air. The residue was dissolved in 0.5 mL of 0.025 M phosphate buffer, pH 7.0, 2 mL of *n*-hexane was added and, after vortexing at the highest speed for 1 min and centrifugation at 3000 *g* for 10 min, the hexane layer was aspirated off. A 100-µL aliquot of the aqueous phase was transferred to an autoinjector vial and 20 µL were injected into the chromatographic system for quantitation.

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Pharmacokinetic Study

Twenty-four healthy adult volunteers (5 females, 19 males) mean age 24.3 ± 5.51 years (range 19 to 45 years), mean body weight 73.2 ± 13.3 kg (48 to 100 kg), and body height 176.3 ± 7.18 cm (range 163 to 189 cm) participated in the study. Written informed consent was obtained from each volunteer prior to participation in the study. Each volunteer received a single oral dose containing 20 mg of enalapril and 12.5 mg of hydrochlorothiazide under fasting conditions. Venous blood samples (6 mL) were drawn into heparinized test tubes immediately before (0) and at 15, 30, 45, 60, 90 minutes and 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10, 12, 16, 24, and 36 hours following drug administration. The blood was centrifuged at 3000 g for 10 min, and the plasma fraction was separated and stored in coded polypropylene tubes at −20°C until analyzed.

RESULTS AND DISCUSSION

Specificity

Individual specificity, in relation to endogenous plasma components, was demonstrated by analysis of a series of randomly selected drug-free plasma samples ($n = 10$). Typical chromatograms obtained from extracts of a drug-free plasma and a plasma sample obtained from a volunteer 6 h after a single oral dose of 20 mg enalapril and 12.5 mg hydrochlorothiazide containing 38 ng/mL of

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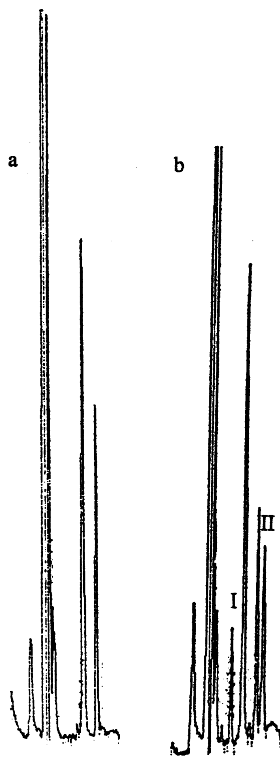

Figure 1. Examples of chromatograms: (a) extract of 1.0 mL drug-free plasma; (b) plasma sample obtained from a volunteer 6 h after a single oral dose of 20 mg enalapril and 12.5 mg of hydrochlorothiazide containing 38 ng/ml of hydrochlorothiazide. Peaks: I = hydrochlorothiazide; II = hydroflumethiazide (internal standard).

hydrochlorothiazide are presented in Figure 1. No endogenous plasma components elute at the retention time of hydrochlorothiazide or internal standard.

Linearity

The linearity of the assay was demonstrated over the concentration range of 5 to 80 ng/mL hydrochlorothiazide by assaying five calibration standards and three quality control samples in triplicate on three separate occasions. Data were obtained through linear regression analysis of peak height ratios vs. concentrations of added hydrochlorothiazide. A weighting factor of 1/concentration was employed. The calibration curves were linear ($r > 0.999$) for concentrations ranging from 5 to 80 ng/mL. Calibration curves were established on each day of analysis.

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System Reproducibility

System reproducibility was determined by assaying seven replicate human plasma samples containing 10, 20, and 40 ng/mL hydrochlorothiazide plus internal standard, which were processed through the assay. The final extracts were pooled in each case, transferred into seven autoinjection vials, and were injected into the HPLC system. System reproducibility, expressed as the coefficient of variation (CV%) and based on absolute peak height, was 2.2, 3.0, and 1.7% ($n=7$) for 10, 20, and 40 ng/mL hydrochlorothiazide concentrations, respectively. Mean reproducibility for the internal standard was 0.9% ($n=21$).

Accuracy and Precision

Assay precision and accuracy were determined by assaying three quality control samples in triplicate on three separate occasions, at each of three concentrations (12, 23, and 45 ng/mL). Concentrations of hydrochlorothiazide in quality control samples were determined by application of the appropriate standard curve obtained on that occasion. Assay precision for hydrochlorothiazide was 9.4% based on coefficient of variation values (CV%) of 9.4, 5.4, and 2.7% for samples containing 12, 23, and 45 ng/mL, respectively. Assay accuracy, assessed by calculating the estimated concentrations as a percent of the nominal concentrations, was better than 97.8%.

Absolute Recovery

The absolute recovery of hydrochlorothiazide and internal standard was assessed by direct comparison of absolute peak heights from extracted vs. non-extracted samples, by using six replicate plasma samples at each of three hydrochlorothiazide concentrations plus the appropriate amount of internal standard. The mean recoveries for hydrochlorothiazide were 65.2 ± 6.1, 63.3 ± 2.9, and $61.4\% \pm 3.7\%$ at the 10, 20, and 40 ng/mL concentration, respectively ($n=6$). Mean recovery of internal standard was $80.0\% \pm 3.2\%$ ($n=18$).

Limit of Quantitation

The limit of quantitation, defined as the lowest concentration on the calibration curve at which both accuracy and precision should be within 20%, was deemed to be 5 ng/mL using a 1.0 mL plasma sample, whose precision and accuracy were well within the proposed criteria.

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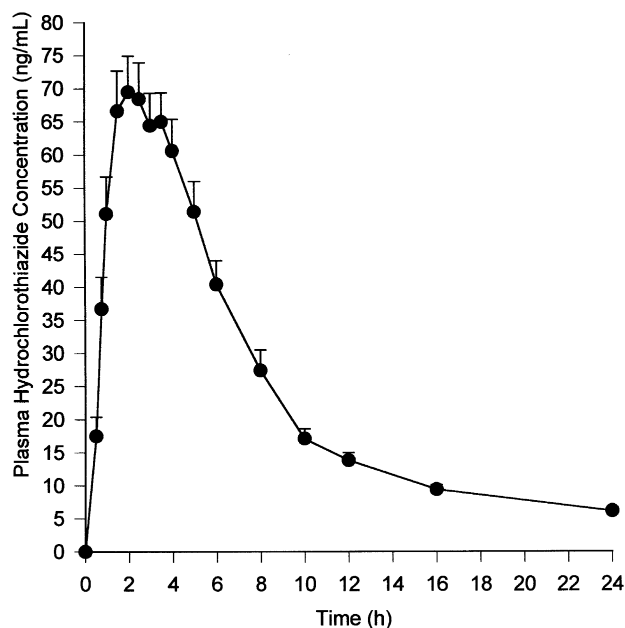

Figure 2. Mean ± SEM plasma concentration-time profile for hydrochlorothiazide following a single oral dose containing 20 mg enalapril and 12.5 mg hydrochlorothiazide to 24 healthy adult volunteers.

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Application to Pharmacokinetic Study

The present method was used to determine the plasma concentrations of hydrochlorothiazide. Figure 2 illustrates the mean ± SEM plasma concentration-time profile of hydrochlorothiazide, following an oral dose containing 20 mg enalapril and 12.5 mg hydrochlorothiazide to 24 healthy volunteers.

CONCLUSIONS

This paper describes a simple, rapid, sensitive, specific, accurate, and precise procedure for the determination of hydrochlorothiazide, suitable for the analysis of large numbers of human plasma samples. The assay was validated to meet the requirements of pharmacokinetic or bioequivalence studies. The method is suitable for routine analysis of hydrochlorothiazide in human plasma at concentrations between 5 and 80 ng/mL.

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