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New sensitive method for the determination of hydrochlorothiazide in human serum by high-performance liquid chromatography with electrochemical detection

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Abstract

A HPLC method with a very sensitive electrochemical detection has been developed for determining the diuretic agent hydrochlorothiazide (HCT) in serum of volunteers to whom a single dose of the fixed combination of 12.5 mg HCT and 25 mg triamterene was administered. In the present method samples (0.2 ml serum, pH 7) were purified by extraction of HCT into 5 ml *tert.*-butyl-methyl ether. The separated organic phase was spiked with *p*-aminobenzoic acid (PABA) standard. The separation was performed on a reversed-phase C_{18} column using phosphate buffer–acetonitrile (90:10). A coulometric cell was used to measure HCT and an ultraviolet detector for PABA. The limit of quantitation for serum samples of only 200 μl was 5 ng/ml (i.e. 60 pg HCT/injection) with a good reproducibility (1–8%). Short retention times were found: 1.2 min for PABA and 5.8 min for HCT.

Keywords: Hydrochlorothiazide

1. Introduction

The diuretic drug hydrochlorothiazide (HCT) is used mainly for treatment of mild to moderate hypertension and is usually administered together with other drugs. Gas chromatographic methods for the analysis of HCT are sensitive but not very suitable for mass analyses [1,2]. Liquid chromatographic methods exist for the quantitation of HCT, however, most assays are time consuming or not sufficiently sensitive [3–8]. To compare two formulations of the fixed combination of 25 mg triamterene and 12.5 mg HCT and for determination of HCT in human serum, a new rapid sensitive HPLC method with a single extraction step and electrochemical detection was developed. In the coulometric cell essentially 100% of the species of interest is oxidised, i.e. the observed signal is much higher than in an amperometric cell where only 10–15% of the analyte is oxidised. The coulometric cell used contains two independently controlled working electrodes in series. At the first one almost all compounds except HCT are captured by oxidation at a lower potential than used for HCT at the second electrode (screen mode).

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2. Experimental

2.1. Chemicals

HCT was kindly provided by Arzneimittelwerk Dresden (Dresden, Germany). Acetonitrile LiChrosolv (for chromatography), chloroform LiChrosolv (for chromatography), methanol LiChrosolv (for chromatography) *tert.*-butylmethyl ether and disodium hydrogen phosphate–$2H_2O$ LiChropur, potassium dihydrogen phosphate for molecular biology were purchased from Merck (Darmstadt, Germany). Pure 4-aminobenzoic acid was obtained from Chemapol (Prague, Czech Republic). Pure water was obtained using the ion exchange system RS 40 E, SG Ionenaustauscher (Barsbüttel, Germany).

2.2. HPLC method

Apparatus and chromatographic conditions

The HPLC system (Shimadzu, Kyoto, Japan) was set up with two LC-10AS pumps, an electrochemical detector (ESA Coulochem II, Bedford, MA, USA) with a coulometric cell (5011), a SPD-10A spectrophotometric detector, a SCL-10A controller, a SIL-10A auto-injector, equipped with a 50-μl loop, a column oven CTO-10AC and a six-channel data system D450-MT2 (Kontron, Neufahrn, Germany). A reversed-phase column (RP 18, 125 × 4 mm I.D. 5 μm; endcapped, LiChroCART HPLC-cartridge; Merck, Darmstadt, Germany) was used for the analysis and the column was maintained at a temperature of 40°C and equilibrated with the mobile phase. The mobile phase was phosphate buffer (0.0075 mol/l, pH 7.3)–acetonitrile (90:10, v/v). The solvent flow-rate was 0.8 ml/min. The electrochemical detector worked in the screen mode. Undesirable or interfering compounds were captured at the first electrode set at a potential of +450 mV and the desired compound HCT was measured at the second set at +630 mV and a sensitivity range of 200 nA full scale. The standard *p*-aminobenzoic acid (PABA) was measured in series with the UV detector at 254 nm (0.5 AU full scale). Short retention times were found: 1.2 min for PABA and 5.8 min for HCT. Standards and control samples were assayed with each series of samples. For reconditioning the electrodes a potential of −400 mV was used overnight.

2.3. Sample preparation

Extraction procedure

The samples of 0.2 ml serum and 20 μl phosphate buffer pH 7.0 (1 mol/l) in 10-ml glass tubes were extracted with 5 ml *tert.*-butylmethyl ether. The mixture was shaken for 20 s (Heidolph-Mixer). After centrifugation for 10 min at 2500 *g* the organic phase was transferred into a 10-ml glass tube containing 200 ng of PABA in 10 μl of methanol and evaporated to dryness at 80°C in a vacuum centrifuge (Jouan Evaporator centrifuge RC 10-20). The residue was redissolved in 180 μl of the mobile phase and a 30-μl aliquot was then injected onto the column.

Standard solutions

Stock solutions of HCT and PABA were prepared by dissolving each of the substances in methanol to a final concentration of 1 mg/ml. Working solutions were obtained by further dilution of the stock solutions with methanol.

Samples

Serum samples were obtained from healthy volunteers during pharmacokinetic studies with a single dose of the fixed combination of 25 mg triamterene and 12.5 mg HCT.

3. Results and discussion

3.1. Chromatography

Symmetrical peaks were observed for HCT and PABA. Typical chromatograms are illustrated in Figs. 1a, b and Figs. 2a–c. The retention times of HCT and PABA were 5.8 and 1.2 min, respectively. The overall chromatographic run time was 7.0 min.

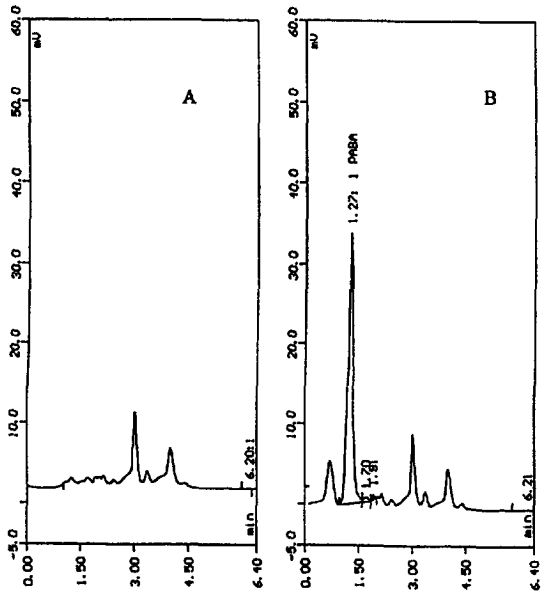

Fig. 1. Chromatograms of serum samples, UV detection. (A) Blank serum; (B) blank sample with PABA. Scale of the ordinate: 1000 mV = 0.5 AU.

3.2. Validation

The linearity and the precision were tested using spiked serum samples. The linearity of the method was confirmed in the range of 5–150 ng/ml using a 0.2-ml sample. The precision of the method was assessed by determination of six concentrations in six independent series of samples as shown in Table 1. The lower limit of quantitation, i.e. a coefficient of variation <10% for six repeated measurements, is 5 ng/ml (60 pg HCT/injection).

Human serum was collected from 16 healthy

Table 1
Precision of the analytical method for HCT

Concentration added (ng/ml)	Concentration found [mean ± S.D. (ng/ml)]	Coefficient of variation (%)
5	5.01 ± 0.42	8.38
10	9.51 ± 0.67	7.08
20	19.64 ± 1.13	5.74
50	49.43 ± 1.31	2.66
100	104.72 ± 2.07	1.97
150	147.15 ± 1.60	1.09

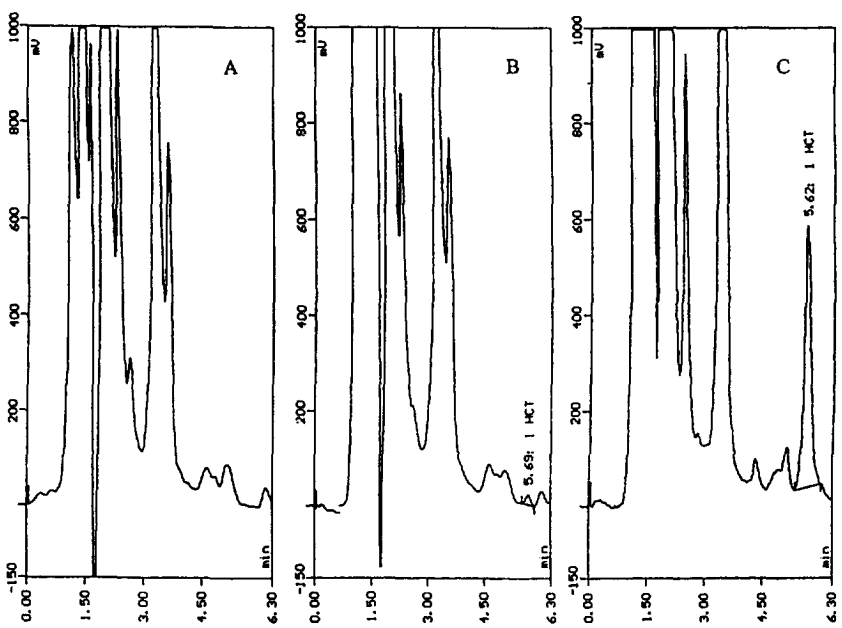

Fig. 2. Chromatograms of serum samples, electrochemical detection. (A) Blank serum; (B) serum spiked with 5 ng/ml HCT; (C) serum sample of a volunteer with 113 ng/ml HCT. Scale of the ordinate: 1000 mV = 200 nA.

volunteers and screened for interference at the retention times of HCT and the volume standard PABA. Figs. 1a and 2a show representative chromatograms of a processed serum blank and indicate that no endogenous compounds exist at the retention times of HCT or PABA. A recovery of 90% of HCT from the serum matrix was found independent of the concentration in the range from 10 to 100 ng/ml. HCT has been shown to be stable in serum maintained at −20°C for up to six months and at ambient temperature for 24 h. Stability has also been established through two freeze–thaw cycles for spiked serum samples.

Day-to-day precision data were obtained over a period of 32 working days by taking aliquots of serum with 8 ng/ml, 30 ng/ml and 150 ng/ml HCT, respectively, and processing them daily. Low coefficients of variation were found: 10.5% (lowest concentration), 5.4% (middle concentration) and 5.6% (highest concentration).

4. Conclusions

This HPLC method with an electrochemical detector with a coulometric cell was found to be selective, sensitive and robust for the measurement of HCT in serum. Furthermore, the assay is fast and requires a relatively simple sample preparation. A large number of samples can be processed daily. This assay allows the quantitation of HCT serum levels for at least 24 h following a single 12.5-mg oral dose of HCT. To increase the sensitivity it is possible to increase the sample volume and/or the sensitivity of the electrochemical detector.

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