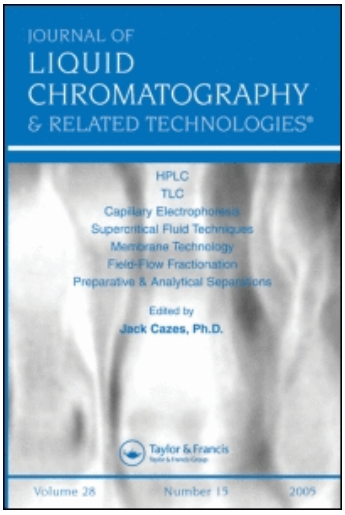

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HIGH PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF HYDROCHLOROTHIAZIDE (HCT) IN PHARMACEUTICAL PREPARATIONS AND HUMAN SERUM AFTER SOLID PHASE EXTRACTION

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ABSTRACT

A rapid, accurate, and sensitive method has been developed for the quantitative determination of hydrochlorothiazide (HCT), an antihypertensive-diuretic agent. A Nucleosil C_{18} 100x4.6 mm, 5 μm analytical column was used with a mixture of CH_3CN-1% acetic acid, at a volume ratio 20:80. Detection was performed with a variable wavelength UV-visible detector at 270 nm, resulting in detection limits of 0.2 ng per 20 μL injection.

For the quantitative determination, hydroflumethazide (HFM) was used as internal standard at a concentration of 4.08 ng/μL. A rectilinear relationship was observed up to 20 ng/μL. Analysis time was less than 6 min.

The statistical evaluation of the method was examined, performing intra-day (n=8) and inter-day calibration (n=8) and was found to be satisfactory with high accuracy and precision results.

The method was applied to the direct determination of HCT in pharmaceutical preparations (tablets) and biological fluids (human serum). Solid phase extraction was used for sample clean-up and analyte retention using Bakerbond C_{18} cartridges. Recovery of HCT in spiked samples was 100.3 ± 0.92% over the range of 0.5-5 ng/μL. No interferences were observed in the assay from other drugs which might be concurrently present during hydrochlorothiazide therapy, such as captopril. Endogenous compounds of human serum did not interfere.

INTRODUCTION

Hydrochlorothiazide (6-chloro-3,4dihydro-7-sulfanyl-2H-1,2,4-benzothiadiazine-1-1-dioxide) has been used as antihypertensive agent due to its diuretic action. It can be prescribed alone, as the sole therapeutic agent, or in combination with other antihypertensive agents, to enhance their effectiveness.[1] Side effects as hyperglycemia, hyperuricemia, hyponatremia, and hypokalemia necessitate the monitoring of serum hydrothiazide level in order to obtain optimal therapeutic response. It is administered in dosages in the range of 25-250 mg/day. After oral administration, HCT is rapidly absorbed and peak plasma concentration occurs at 2 h.[2,3]

To support HCT clinical studies (bioequivalence, bioavailability, interaction), a quantitative analytical method with specificity, selectivity, and sensitivity is required to measure low levels of HCT concentration in biological fluids following low dose administration of HCT to human subjects. Several methods have been reported in the literature to quantify the drug in biological fluids. Among them, colorimetry, TLC, GC, and various forms of high-performance liquid chromatography are included. The former lack specificity and sensitivity, while some of them require tedious sample preparation, clean-up, and derivatization procedures. The latter are preferable as offering selectivity, sensitivity, and robustness.[1,4,5]

Some of the LC methods reported in the literature require large plasma volumes when applying liquid-liquid extraction procedures for the isolation of the drug from the biological matrix.[2,5] Complex, multi-step sample preparations involving derivatizations are followed by gas chromatographic

separations and quantification. These sample preparations are lengthy and time consuming; consequently, these methods are not very suitable for mass analyses.[1,6]

Segmented-stream, continous-flow systems have been used as sample preparation devices for the liquid chromatograph, for quantifying HCT in blood plasma and serum.[7] First derivative UV-spectrometric determination has been reported by some researchers.[8,9] Detection techniques reported in the literature include fluorescence detection,[3] electrochemical detection,[10] and UV detection at several wavelengths.[5,11] Robotics have also been adapted by some researchers requiring automated methods and highly specific equipment.[3]

In the present work, a solid-phase extraction procedure is developed in order to isolate HCT from low volume serum samples and a rapid, sensitive, and reproducible HPLC method is presented to support clinical pharmacokinetic studies.

EXPERIMENTAL

Instrumentation

A Shimadzu (Kyoto, Japan) LC-9A pump was used to deliver the mobile phase to the analytical column, Nucleosil C_{18} 100 x 4.6 mm, 5μm (Rigas Labs, Thessaloniki, Greece). Sample injection was performed via a Rheodyne 7125 injection valve (Rheodyne, Cotati California,U.S.A) with a 20 mL loop. Detection was achieved by an SSI 500 UV-Vis detector (SSI, State College, PA, U.S.A.) at a wavelength of 270 nm and a sensitivity setting of 0.002 AUFS. A Hewlett-Packard (Avondale, PA, U.S.A.) HP3396 Series II integrator was used for quantitative determination of eluted peaks.

A glass vacuum-filtration apparatus, obtained from Alltech Associates, was employed for the filtration of the buffer solution, using 0.2 μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany). Degassing of solvents was achieved by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany) prior to use. A Glass-Col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the sample pretreatment.

The SPE assay was performed on a Vac-Elut vacuum manifold column processor purchased from Analytichem International, a division of Varian (Harbor City, USA). All evaporations were performed with a 9-port Reacti-

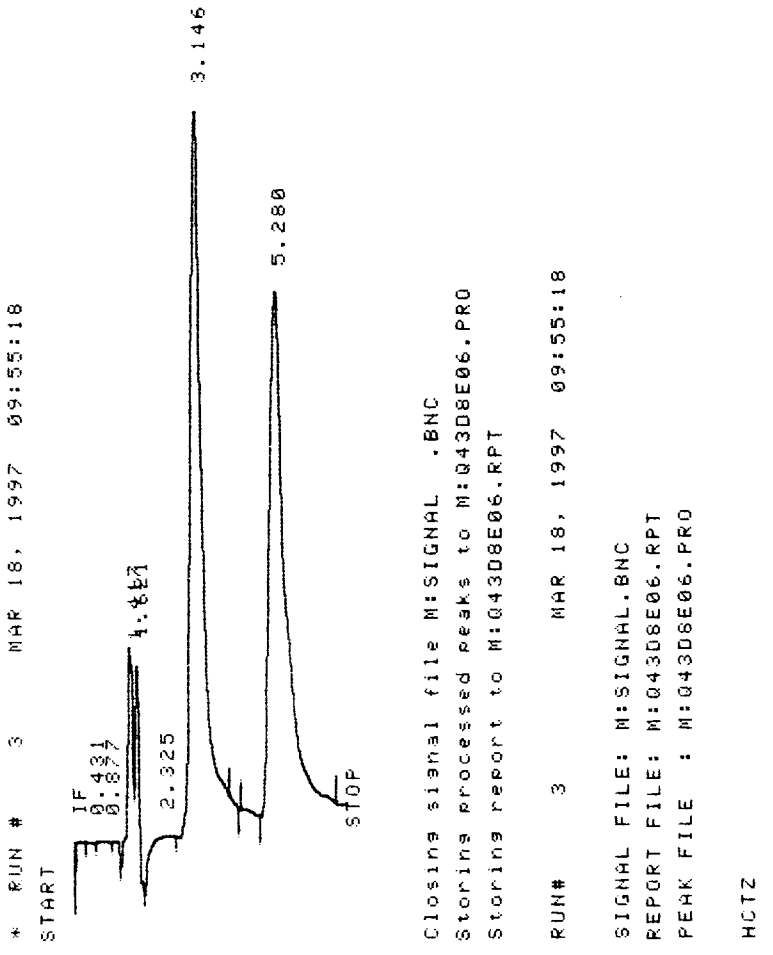

Figure 1. Chromatogram of standard solutions of 3.0 ng/μL HCT (3.146 min.) and 4.08 ng/μL HFM (5.280 min.). Chromatographic conditions are described in the Experimental section.

Vap evaporator (Pierce, Rockford, IL, USA). The UV spectrum of HCT for selecting the working wavelength of detection was taken using a Varian DMS 100S UV/VIS double-beam spectrophotometer. All computations were achieved using a VIP 312 computer.

Materials and Reagents

HCT and HFM were purchased from Sigma Chem. Co. (St. Louis, MO, USA). HPLC gradient grade methanol and acetonitrile were obtained from Lab-Scan (Lab-Scan Analytical Sciences, Ltd, Dublin, Ireland). Glacial acetic acid, p.a., was from Merck (Darmstadt, Germany). Bis de-ionised water was used throughout analysis. Solid phase extraction cartridges, Bakerbond C_{18}, were from J. T. Baker (A Division of Mallinckrodt, Baker B.V. Deventer, Holland). The mobile phase was vacuum filtered before use through 0.2 μm membrane filters (Schleicher-Schuell, Dassel, Germany).

Standard Solutions

Stock solutions of HCT were prepared in methanol and stored refrigerated at 4^0C. These solutions were found to be stable for at least one month. Working methanolic solutions were prepared from stocks at concentrations: 0.5, 1.0, 2.0, 3.0, 5.0, 8.0, 10.0, 15.0 and 20.0 ng/μL. Methanolic solution of internal standard (HFM) was added at a concentration of 4.08 ng/μL.

Chromatographic Conditions

Peak areas of HCT were measured and the ratio to internal standard was compared to that of the calibration standards. The analytical column was maintained at ambient temperature. The isocratic eluent system consisted of 20% acetonitrile and 80% acetic acid (1%).

Retention times revealed were 3.146 min for HCT and 5.280 min for internal standard (HFM), as shown in chromatogram presented in Fig.1. Flow rate 1.3 ml/min led to 100 kg/cm^2 inlet pressure. Sensitivity of the detector was set at 0.002 AUFS.

RESULTS AND DISCUSSION

Performance Characteristics of the Proposed Method

Optimised chromatographic conditions were set and the following analytical characteristics were evaluated:

- Precision and accuracy,
- Working range and detectability,

Table 1

Within-Day Precision and Accuracy for the Analysis of HCT (n=8)

Concentration HCT (ng/μL)	Recovery (%) (x±SD)	RSD (%)
0.5	98±4	4.1
1.0	104±3	2.9
2.0	94±3	3.2
3.0	97±2	2.1

Table 2

Day-to-Day Precision and Accuracy for the Analysis of HCT Over a Period of 8 Consecutive Days

Concentration HCT (ng/μL)	Recovery (%) (x±SD)	RSD (%)
0.5	98±8	8.2
1.0	103±7	6.8
2.0	95±6	6.3
3.0	102±4	3.9

- Analysis time,
- Calibration data,
- Solid-phase extraction,
- Real sample analysis.

Precision and Accuracy

In order to verify the reproducibility, replicate injections of standard solutions at low and high concentration levels were made and peak areas were measured in comparison to the peak area of the internal standard. Statistical evaluation revealed relative standard deviations at different values for eight injections.

The results of intra-day calibration are presented in Table 1. Long term stability was examined during routine separation of the system over a period of eight consecutive days. Results are presented in Table 2.

Working Range and Detectability

Linearity of the calibration curve covers the range between 0.5-20 ng/μL. Detection limit calculated as a signal to noise 3:1 ratio was found to be 0.05 ng/μL, when 20 μL are injected into analytical column. The analytical wavelength 270 nm was chosen for quantitation since it represents a maximum absorbance in the HCT spectrum.

Analysis Time

The analysis time in the proposed method is determined solely by the retention time of the internal standard, which is 5.280 min.

Calibration Data

Calibration of the method was performed by injection of standards covering the entire working range. Each sample was injected five times. Linear correlation (r=0.99616) was obtained using HFM as internal standard at a concentration of 4.08 ng/μL. Calibration curves for HCT are:

$$Y = (0.25842 \pm 0.04207) + (0.27693 \pm 0.00920)X, \text{ where } X = \text{ng}/\mu\text{L}, \text{ and}$$

$$Y = (0.25842 \pm 0.04207) + (0.013846 \pm 0.00046)X, \text{ where } X = \text{ng}.$$

Solid Phase Extraction

100 μL of standard solution were applied to the BakerBond C_{18} SPE cartridges, which were conditioned by flushing with 3 mL MeOH and 3 mL H_2O prior to the addition of sample. After applying the sample, cartridges were washed with 3 mL H_2O and dried by sucking air through them. HCT and HFM were eluted using 3 mL MeOH. The samples were subsequently evaporated to dryness under a gentle nitrogen steam in a 45°C water bath and diluted to 100 μL MeOH. 20 μL of the sample were injected into the analytical column.

Table 3

Precision and Accuracy of HCT Determination in Tablets[a]

Pharm. Prep'n	Inj. Quantity (ng)	Found[b] ±SD (ng)	RSD (%)	Found in Prep'n (ng)	Recovery (%)
Composite 1	20	20.18±0.34	1.7	25.22	100.9
	40	36.72±0.29	0.8	22.95	91.8
	60	58.20±1.22	2.1	24.25	97.0
Composite 2	20	19.68±0.59	3.0	24.60	98.4
	40	38.12±0.57	1.5	23.82	95.3
	60	58.14±0.41	0.7	24.22	96.9
Composite 3	20	19.60±0.18	0.9	24.50	98.0
	40	36.96±0.78	2.1	23.10	92.4
	60	56.70±0.4	0.7	23.62	94.5
Sole Active Ingredient	20	19.62±0.35	1.8	24.52	98.1
	40	39.48±1.26	3.2	24.68	98.7
	60	58.38±0.93	1.6	24.32	97.3

[a] Active ingredient, 25 mg. Composites contain Captopril, 50 mg.
[b] Mean value of six measurements.

Real Sample Analysis

Tablets

Ten tablets of 25 mg (labelled amount) HCT were weighed and finely powdered. A portion of the powdered composite, equivalent to one tablet, was quantitatively transferred to a 100 mL volumetric flask. The contents were diluted to volume with methanol for a concentration of 250 ng/μL according to the labelled amount.

A portion of this solution was transferred to a 100 mL volumetric flask and diluted to volume with methanol. The resulting solution was 50 ng/μL in HCT. Solutions of 1.0 - 2.0 and 3.0 ng/μL, containing HFM in 4.08 ng/μL as internal standard, were prepared after proper dilutions.

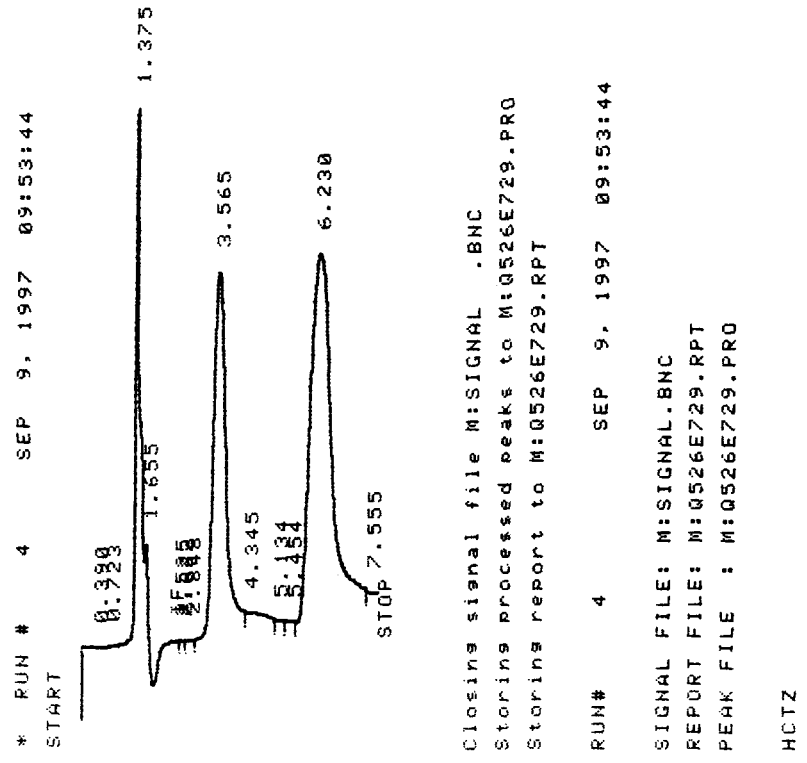

Figure 2. Chromatogram of HCT (2 ng/μL labelled concentration) in composite with captopril. Conditions are described in text.

Table 4

Precision and Accuracy of HCT Determination in Spiked Human Serum Samples

Concentration HCT (ng/μL)	Added (ng)	Found (ng)	RSD (%)	Recovery (%)
0.5	10	9.88±0.24	2.4	98.8
1	20	20.12±0.62	3.1	100.6
2	40	40.14±0.15	3.7	100.4
3	60	60.78±0.51	0.8	101.3
5	100	100.50±1.20	1.2	100.5

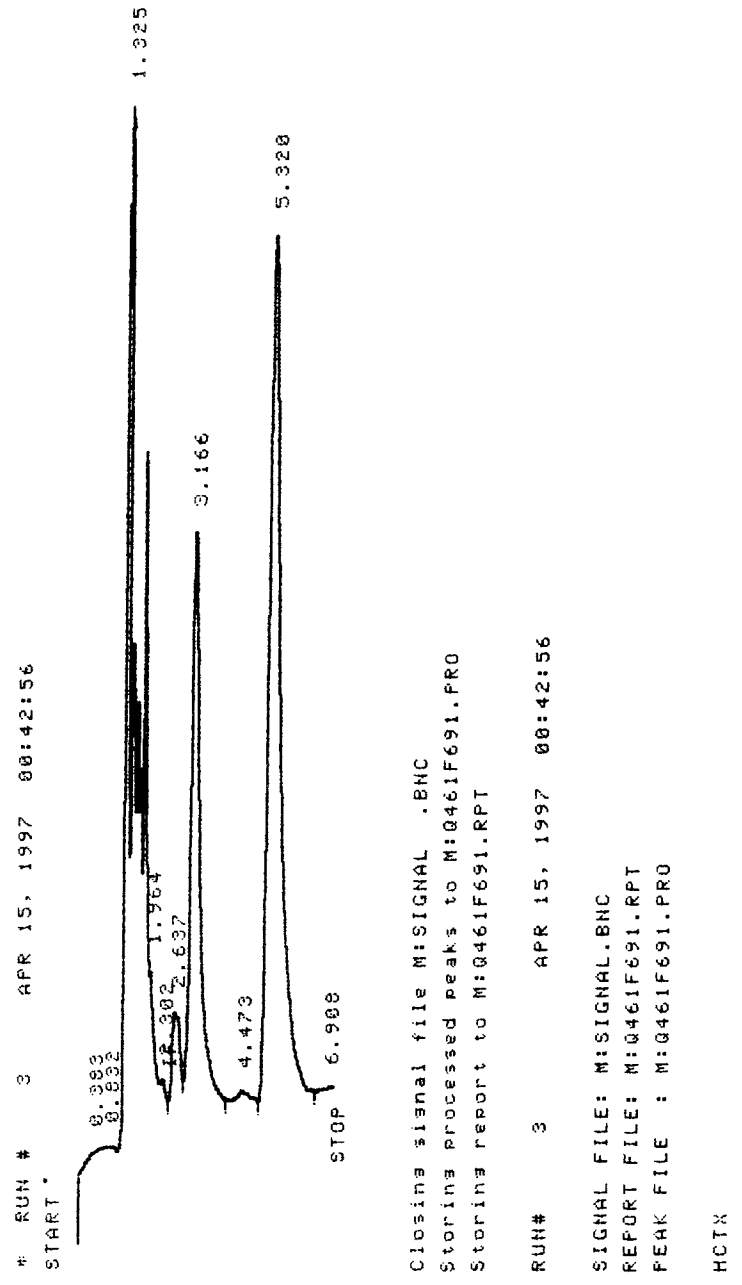

Figure 3. Chromatogram of spiked with HCT (1 ng/μL) human serum sample after SPE. Conditions are described in text.

Six repeated injections of each solution were made and the mean values of the peak area ratios of HCT to internal standard were used for HPLC quantitation. Four pharmaceutical formulations of HCT were analysed. Three of them were composites with captopril, which did not interfere since it does not absorb at the wavelength used for HCT analysis. Results are presented in Table 3.

Human Blood Serum

Aliquots of 100 μL of human blood serum were treated with 200 μL of acetonitrile to precipitate proteins. After 2 min. vortex mixing, 100 μL of mixed standard solution were added to the sample at concentrations: 0.5, 1.0, 2.0, 3.0 and 5.0 ng/μL. The concentration of HFM was 4.08 ng/μL The sample was subsequently centrifuged at 4000 rpm for 15 min and the supernatant was transferred to the SPE cartridge after removal of organic solvent. The sample was subsequently treated according to the procedure described under solid-phase extraction paragraph. Five repeated measurements were made and the mean values of the peak area ratios of HCT to internal standard were used for quantitation. Results are presented in Table 4 (n=5).

CONCLUSIONS

With the proposed method a satisfactory separation of the analytes, extended linear range and a rapid analysis time is achieved. At the retention times of the analytes (HCT and internal standard HFM) no interferences from endogenous compounds were found in chromatograms of pharmaceutical preparations or from extracted spiked human blood serum as can be seen in Figures 2 and 3, respectively.

Captopril present in the three composites of the antihypertensive pharmaceutical formulations did not interfere as it does not absorb at the wavelength used for HCT detection. Peak area ratios of HCT relative to internal standard were linearly related to concentrations ranging from 0.5 to 20.0 ng/μL (or 1-100 ng injected onto the column). The detection limit defined as the quantity producing a signal-to noise ratio of 3 (in terms of peak height) as approximately 1 ng injected on-column.

In order to assess the precision and accuracy of the proposed analytical method, standard solutions of HCT at four concentration levels, with internal standard, were injected 8 times each within the same day and 5 times per day over a period of 8 days.

Thus, within-day repeatability and between-day reproducibility were evaluated. The RSD's and relative recoveries ranged from 2.1 to 4.1% and from 94 to 104 % in within day assay and from 3.9 to 8.2% RSD and 95 to 103% recovery in day-to-day assay.

In pharmaceutical preparations, the recovery of HCT ranged from 91.8-101.3% of the labelled value, which indicates good agreement with HCT amounts as stated at the label (Table 4).

The proposed method also provides a precise and accurate determination of HCT in human blood serum samples. In spiked serum samples, HCT and internal standard HFM were eluted with methanol after SPE clean-up. Recovery of HCT from spiked serum samples ranged from 98.8 to 101.3% with RSD's ranging from 0.8 to 3.7%.

The proposed HPLC method ensures a precise and accurate determination of HCT in pharmaceutical preaparations and blood serum samples. The extraction recovery of the spiked serum after SPE is satisfactory. No interferences from endogenous compounds in the serum matrix or excipients in pharmaceuticals were noticed. Analysis time is approximately 6 min. Small aliquots of serum samples are required, thus providing a suitable analytical method for clinical pharmacokinetic studies.

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