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DETERMINATION OF HYDROCHLOROTHIAZIDE IN SERUM BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A quantitative high-pressure liquid chromatographic method has been developed for the analysis of hydrochlorothiazide in serum in therapeutical concentrations. The method is based on gel filtration of the sera on Sephadex G-15, extraction of the protein-free fraction of the effluent with ethyl acetate and injection of a methanol solution of the drug extract on a reversed-phase column packed with Spherisorb ODS (particle size, 10 μm). The mobile phase is 15% methanol in water. The detection limit is 50 ng/ml of serum. Serum samples from patients receiving hydrochlorothiazide have been analysed by the described method at different hours postdose.

INTRODUCTION

The diuretic drug hydrochlorothiazide (HCT) is widely used in the treatment of hypertension, congestive heart failure and other edematous conditions. Very few methods have been reported for the analysis of plasma levels from patients receiving therapeutic doses of this drug. Spectroscopic methods do not possess the necessary sensitivity and specificity[1]. A gas chromatographic (GC) determination of HCT in blood and plasma, using derivatization and electron-capture detection (ECD), has previously been described[2]. However, this method is very time-consuming since the plasma samples have to be taken through seven extraction steps before GC injection, and the separation time required is more than 20 min. Another method, also based on GC-ECD after extraction steps and derivatization, has recently been reported[3].

Gel filtration has previously been utilized as a preliminary purification method in the determination of drugs from plasma[4]. In this paper we showed that separation on Sephadex as the gel-filtration material is a useful method for the isolation of HCT from plasma proteins, and only one extraction step is necessary to clean up the effluent before liquid chromatography. The simple extraction procedure is a great advantage in the isolation of drugs which have very low solubilities in the common organic solvents.

As GC methods require derivatization of HCT, the application of high-pressure liquid chromatography (HPLC) is preferred in the present paper. The sepa-

ration and quantitation of HCT by means of HPLC and UV detection can be performed without derivatization. The method is also rapid and sensitive, and permits the determination of HCT in human serum at normal dosage levels. The detection limit is 50 ng/ml.

MATERIALS AND METHODS

Apparatus. A Spectra-Physics Model 3500 liquid chromatograph equipped with a rotary valve injector and a UV detector (Model 225) was used. The injector valve was equipped with a standard 10-μl sample loop. The column (3 × 250 mm) was packed with Spectra-Physics Spherisorb ODS (particle size, 10 μm), and the recorder was a Houston Instrument Omni Scribe TM. A Compucorp R344 Statistician was used for the calculations. A Cecil Instruments CE 292 digital apparatus was used for the measurements of the effluents from the Sephadex column.

Reagents. Hydrochlorothiazide (HCT) was obtained from Ciba-Geigy AG. Basel, Switzerland. Trichloromethiazide (Schering Bloomfield, N.J., U.S.A.) was used as an internal standard (IS). Sephadex was obtained from Pharmacia, Uppsala, Sweden. All of the other reagents of analytical grade were supplied ny E. Merck, Darmstadt, G.F.R. 0.05 *M* Tris(hydroxymethyl)aminomethane (TRIS) buffer (pH *ca.* 11) was adjusted to the desired pH value by use of 5 *M* NaOH. The mobile phase was a solution of 15% methanol in distilled water which was filtered through a glass filter disc (G-4) and degassed before use.

Stock solutions of hydrochlorothiazide were prepared by dissolving HCT in 0.01 *M* NaOH to give a concentration of 500 μg/ml and then diluting with distilled water to give solutions of 80 (A), 20 (B), 8 (C) and 2 μg/ml (D). The solutions were stable throughout the experiments. A solution of the internal standard was prepared by dissolving trichloromethiazide in 0.01 *M* NaOH to give a concentration of 500 μg/ml and then diluting with distilled water to give a concentration of 50 μg/ml (E)

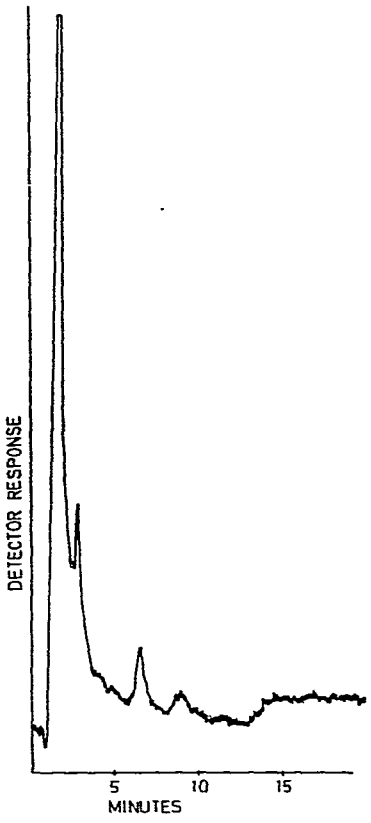
Column preparation and control. The Sephadex G-15 columns were prepared as described previously[4]. An appropriate amount of Sephadex suspension was poured into a glass column (40 × 1 cm I.D.) to give a depth of 12 cm after setting. The column was washed thoroughly with TRIS buffer before use. Columns prepared at different times gave reproducible void volumes and results.

The separation of HCT from serum proteins on the columns was examined by the use of a standard solution of HCT in normal serum having a concentration of 10 μg/ml. 1 ml of this solution was poured on to the dry glass wool of the column and TRIS buffer was added. The effluent containing HCT was determined by collecting fractions of 1 ml. The fractions were diluted with TRIS buffer to 3 ml and assayed spectrophotometrically at 280 nm. The results were compared with a serum blank treated in the same way.

Sephadex filtration and HPLC determination. 1.00 ml of serum was applied to the top of the gel bed. The glass wool was washed with TRIS buffer after the sample had penetrated into the gel matrix, and then TRIS buffer was continually added to the column. After 9.2 ml of effluent had been obtained, a fraction of 4.95 ml was collected in a 5.00-ml calibration flask containing 50 μl of IS solution (E). The pH of the fraction was adjusted to *ca.* 3 with 5 *M* HCl, and the effluent was extracted with 20 ml of ethyl acetate in a mechanical shaker for 15 min. The mixture was then

centrifuged and the ethyl acetate layer was withdrawn into an evaporating tube and taken to dryness at 45° under a stream of nitrogen. The glass walls of the tube were washed with methanol which was then evaporated to dryness. The residue was dissolved in 50 μl of methanol and 10 μl of the resulting solution was injected into the liquid chromatograph. The flow-rate of the mobile phase was 1.2 ml/min and the column pressure *ca.* 810 p.s.i. at room temperature, the wavelength of detection was 280 nm and the sensitivity was set at 0.01 a.u.f.s., the chart speed was 0.5 cm/min.

Calibration graph. The calibration graph was constructed for the concentration range 100–800 ng/ml serum. HCT in solution C or D was added to the effluent after elution of normal serum without any drug. The calibration graph was used for the calculations of the serum samples from patients. The peak-height ratios (drug/internal standard) were plotted *versus* drug concentration in ng/ml serum. Each reference point on the graph was the mean value of six to eight samples analysed by the described method. The relative standard deviations (R.S.D.) were calculated for the different serum concentrations.



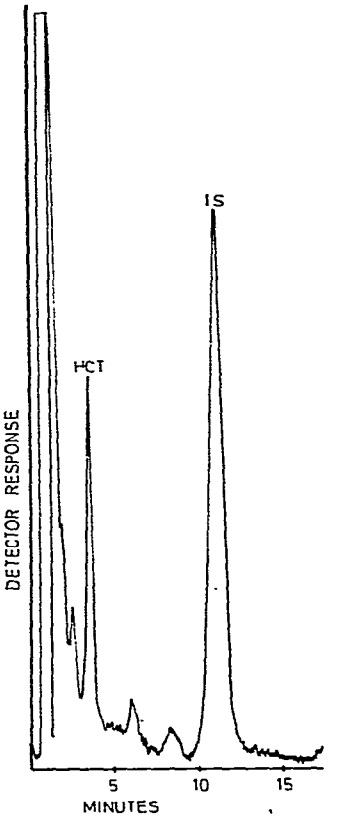


Fig. 1. HPLC of a serum blank. Operating conditions: column (250 × 3 mm), Spherisorb ODS (particle size, 10 μm); mobile phase, 15% methanol in water; flow-rate, 1.2 ml/min; column pressure, 810 p.s.i. at room temperature; detector, UV photometer at 280 nm.

Fig. 2. HPLC of a patient serum containing 458 ng HCT/ml (3 h after dosage). IS, internal standard. Chromatographic conditions as in Fig. 1.

Recovery study. HCT in solution A or B was added to serum to give final concentrations five times the concentrations in the effluent, to correct for the dilution. The samples were then taken through the described method.

RESULTS AND DISCUSSION

Gel filtration and extraction

The gel filtration on the Sephadex G-15 column (12 cm) followed by ethyl acetate extraction at pH 3 gave sufficiently pure extracts for liquid chromatography (Figs. 1 and 2). Experiments with Sephadex G-25 and columns of Sephadex G-15 of different lengths (10 and 15 cm) did not give satisfactory results. Serum was used instead of plasma to give purer extracts. The retention time for HCT was found to be 3.6 min with no interfering peaks in this area. Two unknown compounds from the serum blank shown in Fig. 1 could not be removed by pre-extraction of the buffer eluate with cyclohexane or benzene. A double ethyl acetate extraction did not give higher HCT recovery.

Other available thiazide diuretics (chlorothiazide, polythiazide and bendroflumethiazide) were tried as the internal standard. Only trichloromethiazide was suitable

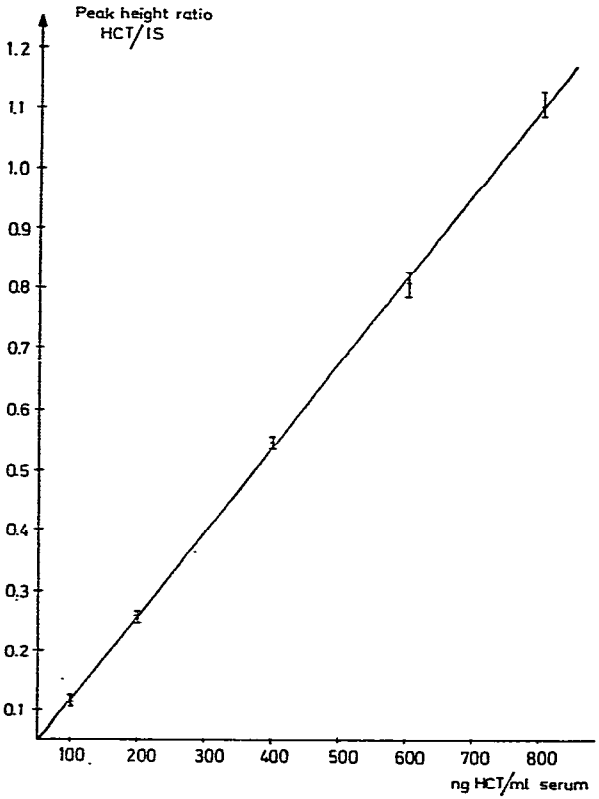


Fig. 3. Calibration graph obtained by adding known amounts of HCT to the effluent after elution of 1 ml of serum on a Sephadex G-15 column.

TABLE I

ACCURACY OF METHOD AND LOOP INJECTION

HTC added x (ng/ml)	*Found, $\bar{x}$ (ng/ml)*	*S.D.*	*R.S.D. (%)*
Assay accuracy			
800	803	10.26	1.28
600	592	12.34	2.08
400	407	5.82	1.43
200	202	4.53	2.26
100	97	6.40	6.40
Loop injection accuracy			
600	583	6.50	1.12
200	196	5.54	2.83

for the analysis of HCT. The retention time for trichloromethiazide was 11 min with no interfering peaks from the serum.

Calibration graph

Fig. 3 shows the calibration graph. The graph ($y = ax + b$) was calculated according to the method of least squares. The correlation coefficient was 0.9992. Table I shows the R.S.D. of the method, and R.S.D. for the loop injection calculated for two different serum concentrations after eight injections of the same extracts. Sera with HCT concentrations four times higher than 600 and 200 ng/ml, were analysed by the described method; the residues were dissolved in 200 μl instead of 50 μl of methanol. With 200 ng HCT/ml serum the R.S.D. obtained was of the same magnitude as that calculated from samples run separately through the method. For 600 ng HCT/ml serum R.S.D. for loop injection was lower than that for samples analysed separately. Table II shows the recovery after Sephadex elution, and the recovery obtained after elution of serum containing a very high HCT concentration (10,000 ng/ml) which was determined spectrophotometrically with serum as the blank. In experiments with spectrophotometric determination it was also shown that increasing the volume of effluent containing HCT did not increase the recovery. The reason why the recovery values are less than 100% is probably that part of the HCT is protein bound.

The degree of protein binding seems to be independent of the HCT concen-

TABLE II

HCT RECOVERY AFTER ELUTION ON SEPHADEX G-15

Concn. (ng/ml)	*Recovery (%)*
100	83
200	77.5
400	72.5
600	71
800	70
10,000	80

tration. The recovery for the sample containing the highest concentration of drug (10,000 ng/ml) lies in the same region as the recovery for a concentration of only 100 ng/ml. The difference between the results for concentrations of 100–200 ng/ml and 400–800 ng/ml is probably due to variable assay accuracy. In the Sephadex system one might expect that the portion of drug associated with protein molecules would be excluded from the gel grains and eluted in the early fraction. The free or unbound drug should appear in a second elution fraction representing the internal volume. Drug–plasma protein bindings both *in vivo* and *in vitro* have been studied using Sephadex filtration[5,6]. This means that analysis of drugs by the proposed procedure would be a valuable tool in the determination of the free fraction of drugs in plasma, providing the rate constants for protein binding allow a separation on the Sephadex columns. More investigations along these lines are necessary.

Detection limit

A detection limit of 50 ng/ml serum was found using the isolation procedure and instrument conditions described. Assay of two series of spiked serum samples gave values of 54 $\pm$ 1 and 77 $\pm$ 2 ng/ml. For sera containing drug concentrations near the detection limit, the final extracts were concentrated to 30–35 μl before injection.

Analysis of patient sera

Sera from five different patients receiving HCT therapy were assayed (Table III). All of the results were calculated from the calibration graph which is based on addition of HCT to protein-free normal sera effluents, and should therefore correspond to the free drug levels. Four of these patients also received other drugs. These drugs were subjected to the HPLC method as controls. Methanol extracts from the tablets and pure drug solutions indicated in Table III were therefore injected. None of these drugs caused interference with the determination of HCT and IS.

TABLE III

HCT LEVELS IN SERA OF PATIENTS

Patient	*Dose (mg)*	*Postdose (h)*	*Serum level (ng/ml)*	*Other medicaments taken*
1	25	16	74	Thyroxin–Natrium
2	25	4.5	659	Aldactone, Mogadon, Lunelax, Primperan
3	75	4	422	Diabinese, Primperan, Torecan
4	50	1.5	165	
		3	458	
		5	130	
		9	87	
5	2 × 25	2.5	243	Lanoxin, Inderal,
		4	*ca.* 137	Diabinese, Meprodifen, Codalgin forte

Patient 5 received HCT two times per day, one morning- and one afternoon-dose. Table III shows hours after morning medication. One of the chromatograms for patient 5 (sample taken 4 h postdose) showed an unknown peak which did not separate completely from the HCT peak. In this case only an approximate value

can be given. Serum from the same patient taken 1.5 h earlier did not show any interfering peak.

Serum from patient 2 showed a surprisingly high HCT level compared with values from the other patients. This patient received HCT as Dichlotride tablets. All of the other patients received HCT as Esidrex. The HCT levels in sera from all of the patients except patient 2, samples being taken at different times after medication, are in line with earlier findings[2].

The described method should be useful for pharmacokinetic and bioavailability studies of HCT. The method is simple and fast. The total time required for chromatographic isolation on a Sephadex column was *ca.* 35 min. However, with a series of columns it was possible to run a large number of samples simultaneously. After this separation only one extraction step is necessary. It should be noted that HCT does not require derivatization before the HPLC detection.

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