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Simple Determination of Hydrochlorothiazide in Human Plasma and Urine by High Performance Liquid Chromatography

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The diuretic drug hydrochlorothiazide (HCT) is used mainly for treatment of mild to moderate hypertension and is usually administered with other drugs. An assay for the determination of HCT in human plasma and urine by high performance liquid chromatography (HPLC) has been developed. Samples were purified by solvent extraction and analysed by reversed phase HPLC with ultraviolet detection, using hydroflumethiazide as the internal standard; plasma was eluted using gradient elution and urine was analysed isocratically. The method is simple to perform, is sensitive (detection limit 0.01 μg/mL in plasma and 0.2 μg/mL for urine); it showed good reproducibility (3–8%). A great number of drugs did not interfere with the assay and the method was used for pharmacokinetic studies in healthy subjects, but samples from patients can also be analysed with high selectivity.

INTRODUCTION

The diuretic drug hydrochlorothiazide (HCT) (6-chloro-3, 4-dihydro-7-sulphamoyl-2*H*-1,2,4-benzothiadiazin-1,1-dioxide) is used mainly for treatment of mild to moderate hypertension; this drug is mostly commercially available in fixed combination products and is usually administered with other drugs (Ings and Stevens, 1983). Interaction of HCT with many drugs has been reported; to study the suspected interaction of the uricostatic drug allopurinol with HCT (Löffler *et al.*, 1989; Vries *et al.*, 1989), an assay for the determination of this drug in plasma and urine was developed.

Several methods for the measurement of HCT in biological fluids have been previously reported—determination by high performance liquid chromatography (HPLC) (Alton *et al.*, 1986; Azumaya, 1990; Barbhaiya *et al.*, 1981; Christophersen *et al.*, 1977; Cooper *et al.*, 1976, 1989; Fett *et al.*, 1991; Henion and Maylin 1980; Koopmans *et al.*, 1984; Shiu *et al.*, 1986; Stewart and Clark, 1986; Van der Meer and Clark, 1987; Yamazaki *et al.*, 1984), gas–liquid chromatography (Lindström *et al.*, 1991; Henion and Maylin 1980).

In the present work plasma and urine samples were purified by solvent extraction and HCT was analysed by reversed phase HPLC and ultraviolet (UV) detection; hydroflumethiazide (HFL) was used an internal standard (I.S.).

EXPERIMENTAL

Preparation of samples. *Volunteers:* During an allopurinol–hydrochlorothiazide interaction study, seven healthy male volunteers (22–28 years) received an oral daily dose of 300 mg of allopurinol for 24 days, and in addition 50 mg hydrochlorothiazide daily from day 11 to day 21. Forearm venous blood was drawn into heparinized tubes at various time intervals and centrifuged immediately; 24 h urines were collected throughout the study (Löffler *et al.*, 1989; Vries *et al.*, 1989); the plasma and urine samples were kept frozen until analysis. *Patients:* blood samples were obtained before the next drug intake and processed as above. Immediately before analysis the samples were thawed at 37 °C and centrifuged.

Extraction procedure for plasma. 1 mL plasma was mixed with 1 mL acetate buffer (0.38 g ammonium acetate in 500 mL aqueous solution and acidified to pH 5.0 with glacial acetic acid), 0.1 mL HFL (0.02 mg/mL methanol; I.S.) 0.2 mL water and 6 mL ethyl acetate; the mixture was shaken for 5 min, and centrifuged for 5 min at 900 g. Five mL of the organic phase was transferred to a clean glass dish and evaporated at 37 °C under a stream of nitrogen. Before analysis, the extract was treated with 100 μL methanol and sonicated twice for 1 min at 37 °C; before filling the vials for the automatic injection, the glass tubes were cooled for 2 h at 2–8 °C to obtain a clear solution.

Extraction procedure for urine. Urine was extracted as described for plasma. The evaporated extract was dissolved directly with 100 μL eluent.

HPLC equipment. A Series HP 1050 System (Hewlett-Packard GmbH, Bad Homburg, Germany) was used for the assay of plasma and urine samples; it consisted of a quaternary pumping system and autosampler; peaks were detected with a variable UV detector (Model SPD-2A) and integrated with a CR-1B integrator–plotter (both Shimadzu GmbH, Duisburg, Germany). The UV detector was set at 280 nm. For the determination of possible interferences with other drugs and peak purity tests, an HP 1090 A System (Hewlett-Packard) with photodiode array detection was used.

HPLC plasma analysis. Chromatographic conditions: 20 μL plasma extract solution was injected into the HPLC system. HPLC column: Nucleosil 100-5 C18 (125 × 4 mm i.d.; 5 μm spherical particles; Macherey Nagel, Düren, Germany); guard column: C18 Corasil (40 × 4 mm i.d.; 35–50 μm; Waters–Millipore, Eschborn, Germany). Eluent: solvent (A)

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25 mL acetonitrile, 1 mL acetic acid in 975 mL water; solvent (B) 500 mL acetonitrile, 1 mL acetic acid in 500 mL water. Solvent flow rate: 1 mL/min. Linear gradient elution settings: 0–16 min (0% B to 64% B); re-equilibration (0%B) for 24 min before the next injection. Peak areas were integrated, and the concentrations were calculated using the corresponding calibration factor.

HPLC urine analysis. Chromatographic conditions: 20 µL of urine eluate were injected into the HPLC system as described for plasma. Elution was isocratic with a solvent containing 120 mL acetonitrile and 1 mL acetic acid in 880 mL water.

Preparation of standards. *Plasma:* A standard stock solution of HCT containing 10 mg in 50 mL water was prepared and maintained for one week at 2–8 °C in the dark; this was diluted 1/50 and 1/500 with water; increasing amounts of the diluted solutions were added to pretreatment plasma to obtain final plasma concentrations of 0, 50, 100, 150 and 200 ng/mL; the internal standard HFL (100 µL HFL; 0.02 mg/mL methanol) was also added to each plasma sample. *Urine:* The same HCT stock solution used for plasma was used to obtain the urine standards; increasing amounts of the stock solution were added to blank urine to obtain concentrations of 0, 5, 10, 15 and 20 µg/mL HCT; the internal standard HFL (100 µL HFL; 0.2 mg/mL methanol) was added to each urine sample.

Calibration curves. Calibration curves for plasma and urine analysis were obtained by processing plasma and urine with known amounts of HCT and HFL as mentioned above, before every analysis series. Least-squares linear regression of the ratio of HCT peak area/HFL peak area vs. added concentrations was used to calculate the calibration factor.

Chemicals. All chemicals were of analytical grade quality. HCT was a gift from MSD Sharp & Dohme GmbH (München, Germany); HFL was obtained from Sigma Chemie GmbH (Deisenhofen, Germany); acetonitrile was of spectroscopic quality (Merck, Darmstadt, Germany).

RESULTS AND DISCUSSION

Interfering substances

The following pure compounds were anlaysed by HPLC under the conditions used for the plasma and urine assays: acebutolol, acenocoumarol, acetylsalicylic acid, allopurinol, ambroxol, amoxicillin, ascorbic acid, atenolol, bendroflumethiazide, benzbromarone, bezafibrate, biperiden, bisacodyl, bromazepam, butizide, caffeine, captopril, cimetidine, ciprofloxacin, clobutinol, clonidine, caffeine, cotinine, diazepam, diclofenac, digitoxin, digoxin, dihydrocodein, dihydroergotamin, diltiazem, doxepin, doxycyclin, enalapril, erythromycin, fenoterol, furosemide, glibenclamide, heparin, hypoxanthine, ibuprofen, indometacine, isosorbidmononitrate, lisinopril, lovastatin, maprotilin, methyldigoxin, methyldopa, metoclopramid, metoprolol, metronidazol, midazolam, naloxone, nifedipine, nicotine, norfloxacine, ofloxacine, oxazepam, oxipurinol, paracetamol, pentoxyfylline, phenacetine, phenazone, phenoxymethylpenicillin, propyphenazone, phenprocoumon, ranitidine, salicylic acid, sotalol, sulphamethoxazole, trimethoprime, terbutaline, theophylline, tilidine, timolol, triamterene, uric acid, verapamil, warfarin, xanthine; purine and pyrimidine bases, nucleosides and nucleotides.

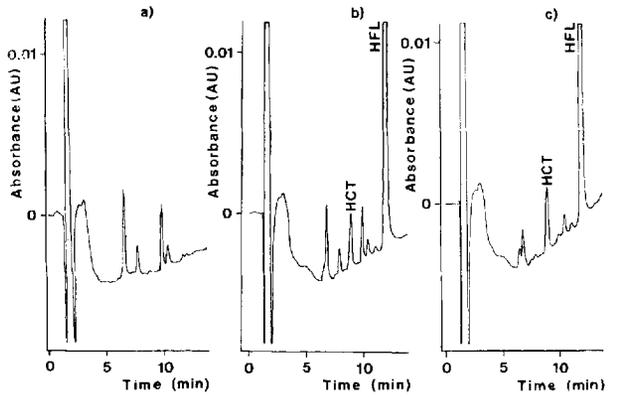

Figure 1. HPLC analysis of plasma extracts: (a) control plasma; (b) control plasma after addition of 150 ng/mL HCT and 2000 ng/mL HFL; (c) plasma sample from a volunteer after a single oral dose of 50 mg HCT (concentrations: HCT 185 ng/mL and 2000 ng/mL HFL).

Amiloride interfered with the internal standard HFL; nevertheless, HCT can be determined using external standard calibration. Under elution conditions for the urine assay, caffeine showed a similar retention time to HCT, and norfloxacine and ofloxacine similar to HFL; however, under the gradient elution conditions as used for plasma analysis, these compounds were readily separated; HCT and HFL showed retention times 9.0 and 12.0 min for plasma and 5.0 and 10.0 min for urine analysis, respectively (Figs. 1 and 2).

The present HPLC hydrochlorothiazide assay differs from those previously reported (see Introduction) in its selectivity, simple extraction procedure and for the analysis of plasma as well as urine samples. Interference from other drugs has not been investigated in most of the reported HPLC methods, although the separation of 23 diuretics was described (Cooper *et al.*, 1989) it was applied for urine screening only. Extraction procedures in most previous methods used multiple liquid extractions or a combination of column and liquid extraction procedures instead of the presently described single step; a direct injection method (Fett *et al.*, 1991) has been described only for hydrochlorothiazide analysis in urine samples but without investigating the possible interferences.

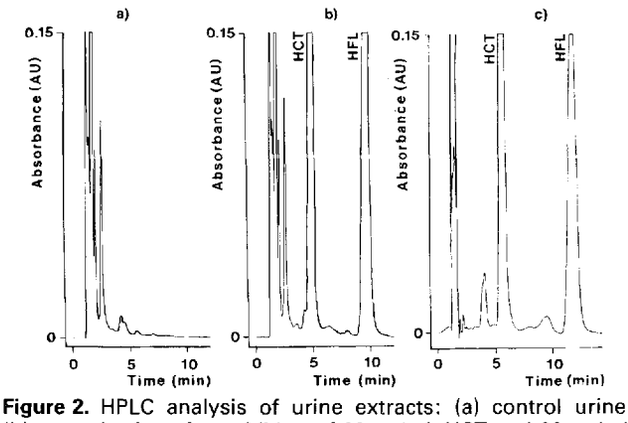

Figure 2. HPLC analysis of urine extracts: (a) control urine; (b) control urine after addition of 20 µg/mL HCT and 20 µg/mL HFL; (c) 0–24 h urine sample from a volunteer after a single oral dose of 50 mg HCT (concentrations: HCT 18 µg/mL and 20 µg/mL HFL).

Plasma analysis

HPLC analysis of HCT in plasma was preceded by extraction with ethyl acetate, as this solvent showed the best recovery for HCT and HFL when compared with other organic solvents; it is essential to treat the extracts as described in the Experimental Section, otherwise the analysis results are very variable. The calculated total recovery was 82% for HCT and 93% for HFL at a concentration of 200 ng/mL; HPLC of various plasma extracts is shown in Fig. 1: Fig. 1(a) is derived from a sample of control plasma, Fig. 1(b) from control plasma after the addition of known amounts of HCT and I.S. and Fig. 1(c) from a volunteer's plasma after 15 days on a daily dose of 300 mg allopurinol and 50 mg HCT; the sample was collected 1 h after drug administration.

Calibration curves were linear in the range 0–400 ng/mL; the limit of detection, defined as 3σ above the measured average blank (MacDougall, 1980) was 10 ng/mL; a within-run precision of 3–8% relative standard deviation was found.

Figure 3 shows the plasma concentration–time profile for HCT from a volunteer after HCT administration.

The HPLC methods described previously (see Introduction) did not show the reproducibility and selectivity required for the assay; the use of chlorothiazide (Barbhaiya *et al.*, 1981; Shiu *et al.*, 1986) as I.S. is not recommendable due to its instability, interference from peaks which coelute and poor chromatographic separation from HCT.

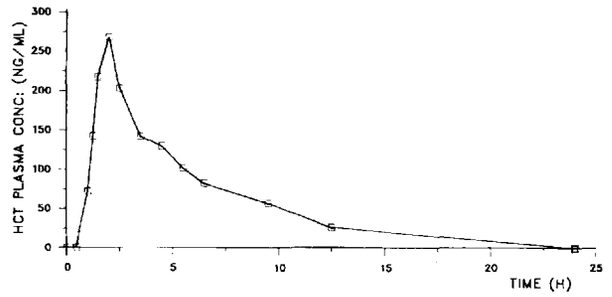

Figure 3. Plasma hydrochlorothiazide concentration–time profile; samples were from a volunteer after a 50 mg single oral dose hydrochlorothiazide.

The present method is time consuming for the dissolution of the extract residue and for HPLC analysis time; however, a single extraction step is used, instead of more complex procedures; a gradient elution was used instead of an isocratic one because of better resolution from interfering peaks. The method showed good precision, sensitivity and selectivity.

Urine HPLC analysis

Extraction of urine samples was similar to that for plasma and isocratic elution was sufficient to separate HCT and HFL from interfering substances. HPLC of various urine extracts are shown in Fig. 2: Fig. 2(a) is derived from a sample of control urine, Fig. 2(b) from control urine after addition of known amounts of HCT and Fig 2(c) from a volunteer's urine after drug administration. The calculated analytical recoveries were found to be 84% for HCT and 90% for HFL; the limit of detection defined as in plasma analysis was 0.2 μg/mL and within-run precision was 3–5% relative standard deviation.

The analysis of urine required shorter HPLC times than plasma, and was adequate for pharmacokinetic and clinical investigations.

The peak purities of HCT and HFL in plasma and urine samples from volunteers after HCT intake were checked by HPLC analysis with photodiode array detection after the extraction step; UV spectra on the ascending, apex and descending portions of the chromatographic peaks were identical to those of the pure compounds.

CONCLUSION

In summary, a specific and sensitive method has been developed for the analysis of HCT in plasma and urine extracts by HPLC with UV detection. It has been used for pharmacokinetic studies and, due to its high selectivity, samples from patients may be analysed without interferences from the large number of drugs commonly used concomitantly.

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