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Note

Sensitive liquid chromatographic method for the determination of hydrochlorothiazide in human plasma

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Hydrochlorothiazide (Fig. 1) is a potent diuretic very frequently prescribed alone or in combination for the treatment of hypertension. Numerous methods of analysis for hydrochlorothiazide levels in various biological matrices have been published over the past fifteen years. Many of the methods focus on the analysis of urine, where hydrochlorothiazide levels are high. Early plasma methods are available which take advantage of the electron-capturing nature of the compound. Complex multi-step sample preparations involving derivatizations are followed by gas chromatographic separations and quantification [1,2]. Sensitivities of these methods approach low ng/ml levels [3,4] but the sample preparations are time-consuming and do not lend themselves to processing the large numbers of samples associated with pharmacokinetic studies in a timely manner. Methods utilizing high-performance liquid chromatographic (HPLC) separations are also available. These methods generally involve less complicated sample preparations but suffer from the lack of sensitivity limitations [5–10]. Sensitivity in the low ng/ml levels has been achievable only by analyzing large sample aliquots [11]. These sample volumes (5 ml per assay) may be prohibitive in studies from which estimates of pharmacokinetic parameters are typically required.

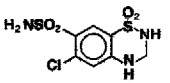

Fig 1. Structure of hydrochlorothiazide

A new sensitive and selective HPLC method is described for the analysis of hydrochlorothiazide in heparinized plasma. The method achieves a quantitation limit of 1.00 ng/ml, utilizing only a 500-μl sample aliquot. A simple, quick sample preparation allows processing and analysis of more than 70 samples per day and plasma levels are quantifiable for up to 48 h following a 12.5-mg oral dose of hydrochlorothiazide.

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EXPERIMENTAL

Materials

HPLC-grade acetonitrile and methanol and reagent-grade glacial acetic acid and triethylamine were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). High-purity methyl *tert.*-butyl ether was obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Heptanesulfonic acid sodium salt and procainamide · HCl were obtained from Sigma (St. Louis, MO, U.S.A.). Hydrochlorothiazide reference standard was from the U.S.P. (Rockville, MD, U.S.A.). Distilled water was further purified and deionized by a Sybron/Barnstead Nanopure II system (Boston, MA, U.S.A.).

Instrumentation and chromatographic conditions

The chromatographic system consisted of an ACS Model 351 isocratic pump, a Varian 9090 autosampler and a Kratos 773 ultraviolet detector. A stainless-steel column (15 cm × 4.6 mm I.D.) was packed with Hypersil ODS, particle size 3 μm (Shandon). The analytical column was preceded by a 2 cm × 4.6 mm I.D. C_{18} prepacked guard column (Supelco Pelliguard). The column was maintained at ambient temperature. Hydrochlorothiazide and procainamide · HCl were determined by UV absorbance at 272 nm (0.003 a.u.f.s.). The mobile phase consisted of acetonitrile–0.007 *M* heptanesulfonic acid sodium salt (18:82, v/v), containing 1% (v/v) glacial acetic acid and 0.035% (v/v) triethylamine, and was delivered at the flow-rate of 0.8 ml/min. Under these conditions, the retention times for hydrochlorothiazide and procainamide · HCl were 5.1 and 10.1 min, respectively.

Biological fluids

Blank human plasma was isolated from heparinized blood drawn from drug-free volunteers. Blood was drawn into evacuated, heparinized collection tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, U.S.A.) and chilled. Samples were centrifuged for 10 min (approximately 1250 *g*) to separate the plasma, which was collected and stored at −20°C until use. Blood samples were also collected from healthy male volunteers after either 12.5- or 25-mg oral doses of hydrochlorothiazide.

Preparation of standards

A stock solution of hydrochlorothiazide was prepared at 100 μg/ml in methanol. Appropriate dilutions of the stock were made with deionized water and the spiking solutions were used to prepare spiked plasma standards at final concentrations of 1.00, 5.00, 10.0, 50.0, 100, 200 and 500 ng/ml. Aliquots of 500 μl of plasma were utilized. Spiked plasma quality control samples were prepared in pools of 20 ml at final concentrations of 2.00 and 70.0 ng/ml, and individual aliquots were frozen. A stock internal standard solution of procainamide · HCl

was prepared at 100 μg/ml and diluted to 20 μg/ml with methanol as the spiking solution. All solutions are stable for up to one month when stored at 4°C.

Sample preparation

Plasma samples were centrifuged (approximately 1250 *g*) for 10 min to compact solids. To 15-ml conical glass centrifuge tubes with PTFE stoppers, 500-μl aliquots of sample were added. Samples were spiked with 25 μl of the internal standard spiking solution and vortexed. To each tube were added 5 ml of methyl *tert.*-butyl ether. Samples were extracted at low speed on a reciprocating shaker for 15 min. After centrifugation for 10 min (approximately 1250 *g*), the organic layer was transferred to a 10-ml conical Reacti-vial. The methyl *tert.*-butyl ether was evaporated under a stream of nitrogen. The residue was reconstituted in 200 μl of mobile phase, and 100-μl aliquots were injected onto the liquid chromatograph under the previously stated conditions.

Data analysis

Data were collected by a VG Multichrom data acquisition system (VG Data Systems, Cheshire, U.K.). Sample concentrations were determined by a response factor fit of the spiked standards based on peak-area ratios of hydrochlorothiazide to internal standard.

RESULTS AND DISCUSSION

Chromatography

A typical blank plasma chromatogram and blank plasma spiked with hydrochlorothiazide (1.0 or 500 ng/ml) and procainamide · HCl (1000 ng/ml) are presented in Fig. 2. A total run time of 14 min allowed separation of the hydrochlorothiazide and internal standard from endogenous plasma components, with retention times of 5.1 and 10.1 min, respectively.

Linearity and quantitation limit

Plasma standards were prepared at 1.00, 5.00, 10.0, 50.0, 100, 200 and 500 ng/ml levels. Plots of the peak-area ratio of hydrochlorothiazide to procainamide · HCl *versus* concentration yielded linear plots fit to the equation $y = 0.003496x - 0.003614$ ($n = 3$, $r = 0.9984$). A signal-to-noise ratio of 4:1 was achievable with the lowest standard, allowing a quantitation limit of 1.00 ng/ml in plasma.

Recovery

Absolute recovery of hydrochlorothiazide through the sample preparation procedure over the entire calibration range averaged 87% [$n = 39$, coefficient of variation (C.V.) = 12.0%]. No concentration effect on recovery was noted. Recovery was determined by injecting unextracted standard at equivalent concen-

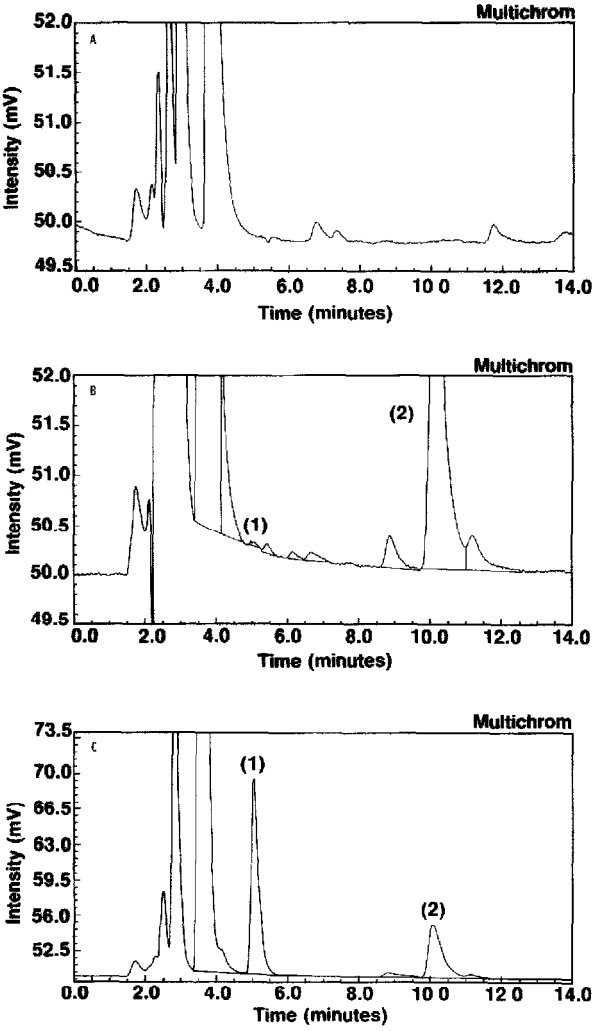

Fig 2 Chromatograms of (A) heparinized plasma blank, (B) heparinized plasma spiked at 1.00 ng/ml hydrochlorothiazide (1) and 1000 ng/ml procainamide (2), and (C) heparinized plasma spiked at 500 ng/ml hydrochlorothiazide (1) and 1000 ng/ml procainamide (2).

trations and comparing the peak areas with those obtained from extracted standards.

Selectivity

The method is specific against aspirin, acetaminophen, ibuprofen and their metabolites. Screening of plasma blanks drawn from twenty drug-free volunteers yielded no endogenous peaks which interfered in the chromatogram.

TABLE I

RESPONSE FACTOR INTER-DAY PRECISION

Concentration (ng/ml)	n	Average response factor (× 1000)	C V (%)
1.00	4	3 26	2 9
5.00	5	3 38	15.3
10.0	5	2.81	9 7
50.0	5	3.27	13 0
100	6	3.05	13 8
200	6	3.52	7.6
500	6	3.47	11.5

Precision and accuracy

Response factors (peak-area ratios/concentration) calculated at each level over three days exhibited a mean within-day C.V. of 6.4% ($n=2$ at seven concentrations) and a mean between-day C.V. of 14.2% ($n=6$ at seven concentrations). Spiked plasma standards averaged 99.5% of theory with a precision (C.V.) of 8.5% over all concentrations over three days. These data are presented in Tables I and II. Quality control samples spiked at 2.00 and 70.0 ng/ml were assayed on each of three days and averaged 92.5 and 99.0% of theory, respectively. These data are presented in Table III.

Stability

Hydrochlorothiazide has been shown to be stable in human plasma maintained at −20°C or lower for up to six months. Stability has also been established through three freeze–thaw cycles for spiked plasma samples.

TABLE II

INTER-DAY STANDARD PRECISION AND ACCURACY

Concentration added (ng/ml)	n	Concentration found (mean ± S.D.) (ng/ml)	C.V (%)	Percentage deviation from theory
1.00	4	0.99±0.11	11.1	−1.0
5.00	5	5.00±0 33	6.6	0 0
10.0	5	9.19±0.86	9.4	−8 1
50.0	6	50.6 ±2.71	5.4	1 2
100	6	92.0 ±2.94	3.2	−8.0
200	6	217 ±17 7	8 2	8 5
500	6	516 ±19.8	3 8	3 2

TABLE III

INTER-DAY QUALITY CONTROL PRECISION AND ACCURACY

Concentration added (ng/ml)	*n*	Concentration found (mean ± S.D.) (ng/ml)	C V (%)	Percentage deviation from theory
2.00	6	1.85±0 14	7 5	−7 5
70 0	5	69 3 ±2 81	4 1	−1 0

Applications

Healthy male volunteers received either 12.5- or 25.0-mg oral doses of hydrochlorothiazide. Heparinized blood samples were drawn immediately prior to dosing and at twelve subsequent time points. Samples were immediately centrifuged and plasma samples stored at −20°C or lower until analyzed. All samples were analysed by the method presented here. Typical concentration–time profiles after a 12.5- and 25-mg oral dose of hydrochlorothiazide are shown in Fig. 3.

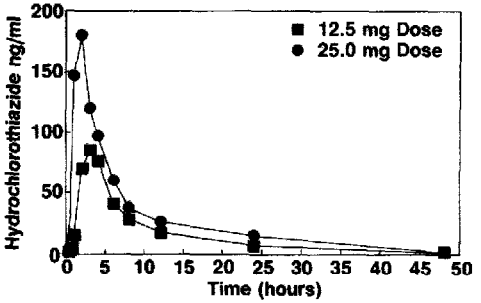

Fig. 3 Representative concentration–time profiles of subjects after a 25- or 12.5-mg oral dose of hydrochlorothiazide.

CONCLUSIONS

The method developed for the analysis of hydrochlorothiazide in human plasma is specific and sensitive, affording a rugged quantitation limit of 1.00 ng/ml. The method is fast and reliable and has been used to monitor plasma levels in clinical trials generating over 2500 samples. Utilizing a relatively simple sample preparation procedure, large numbers of samples can be processed daily (approximately 80–90). Over 1000 sample injections have been made on a single column with no loss of chromatographic integrity. This method allows the quantitation

of plasma levels of hydrochlorothiazide for at least 24 h and in many cases 48 h, following a single 12.5-mg oral dose, allowing for complete characterization of the resulting plasma concentration profile.

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