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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR HYDROCHLOROTHIAZIDE IN HUMAN URINE

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SUMMARY

A high-performance liquid chromatographic assay was developed for the quantitative determination of hydrochlorothiazide (HCT) in human urine. Reversed-phase separation of HCT and the internal standard, trichloromethiazide (TCMT), was accomplished on a 300 × 3.9 mm μBondapak Phenyl column. Following solvent extraction, concentrations of HCT as low as 0.25 μg/ml in urine were quantified by UV detection at 280 nm. Detector response (peak-area ratio of HCT to TCMT) was linear to 50 μg/ml. No interferences were observed in the extracts obtained from drug-free urine nor from several antihypertensive agents which are commonly co-administered with HCT. This method has been routinely employed in bioavailability studies evaluating a variety of formulations as well as characterizing the pharmacokinetics of this drug from urinary excretion data.

INTRODUCTION

Hydrochlorothiazide, 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (HCT), is a diuretic agent shown to be effective in the treatment of hypertension [1]. Literature findings [2—7] have demonstrated that urinary excretion data may be used to assess the bioavailability of various formulations containing this thiazide. In order to support a multitude of clinical bioavailability/bioequivalency studies, a quantitative method was needed which was both specific for HCT in the presence of labetalol and other antihypertensive agents as well as sensitive enough to measure concentrations of HCT in urine up to 48 h following the administration (per os) of HCT (25 mg) to human subjects. Several methods which employ either colorimetry [8] or high-performance liquid chromatography (HPLC) [5, 9–11] have been reported for the quantification of HCT in human urine. The reported inter-

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ference by labetalol in the HPLC assay described by Soldin et al. [9] precluded use for our specific application. Methods [5, 9, 10] which have a lower limit of quantitation approximating 1—2 μg/ml were not sufficiently sensitive for the determination of urinary drug levels beyond 36 h in many subjects given a single 25-mg oral dose of HCT. In order to fully characterize the terminal elimination phase of HCT in urine, an assay with greater sensitivity (ca. 0.25 μg/ml) was desired. The HPLC assay reported by Koopmans et al. [11] offers improved sensitivity (ca. 0.5 μg/ml); however, expensive ion-pair reagents and a column oven (38°C) were required for chromatographic separation. In addition, no information was provided about the selectivity of this method in the presence of other antihypertensive agents which are commonly co-administered. An HPLC assay for the determination of HCT in urine was therefore developed which addressed the specific needs of our clinical program. The development and validation of this method are described herein.

EXPERIMENTAL

Apparatus

Analyses were performed on an HPLC system consisting of a WISP (Model 710B) automatic injector (Waters Assoc., Milford, MA, U.S.A.), a Waters M6000A pump and a Lambda-Max Model 480 LC spectrophotometer (Waters Assoc.). Chromatograms were traced on a Varian Model 9176 (Varian Assoc., Palo Alto, CA, U.S.A.) strip-chart recorder while peak-area integration was performed by a Hewlett-Packard Series 3350 (Avondale, PA, U.S.A.) computer interfaced with the detector by employing a Model 18652A (Hewlett-Packard) A/D converter.

Reagents and solvents

Hydrochlorothiazide, the internal standard trichloromethiazide (TCMT), chlorothiazide, labetalol · HCl, propranolol · HCl, pindolol, metoprolol and timolol were used as received. All other chemicals except ethyl acetate and methanol (OmniSolv, MCB, Cincinnati, OH, U.S.A.) were reagent grade.

Chromatographic conditions

Reversed-phase separations were accomplished at ambient temperature on a 300 × 3.9 mm μBondapak Phenyl (10 μm particle size) column (Waters Assoc.) using a mobile phase consisting of 0.1 *M* potassium dihydrogen phosphate (pH 7.15)—acetonitrile—tetrahydrofuran (85:10:5). The solvent mixture was prepared daily, filtered through a Nylon-66 membrane (0.45 μm) and degassed under reduced pressure before use. The flow-rate (2.0 ml/min) generated a back pressure of approximately 150 bar. The analytical column was protected by a guard column (30 × 3.9 mm) dry-packed with Phenyl Corasil (Waters Assoc.), a pellicular (40 μm particle size) support.

Instrument settings

The output voltage (1 V per 1 a.u.f.s.) from the Model 480 detector was attenuated with a variable input adapter to protect the A/D converter from

saturation. The detector sensitivity at 280 nm was adjusted to 0.05 a.u.f.s. for strip-chart recording (10 mV) of each chromatographic analysis.

Standard solution preparation

HCT (50 mg) was dissolved in methanol and diluted to 100 ml in a volumetric flask. A solution of the internal standard, TCMT, was similarly prepared by dissolving 50 mg in methanol and then diluting to volume in a 100-ml volumetric flask. Subsequent dilutions with methanol were designed so that the desired amount of either drug could be conveniently delivered in 0.1 ml by using automatic pipetting devices.

Extraction procedure

An aliquot (1 ml) of human urine was transferred to a 15-ml test tube (16 × 125 mm) fitted with a PTFE-lined screw cap. After the addition of internal standard (5 μg), each urine sample was diluted with 1 ml of 0.1 *M* sodium bicarbonate and extracted with a single 5-ml volume of ethyl acetate by agitation on an Eberbach (Ann Arbor, MI, U.S.A.) reciprocal shaker for 10 min. Samples were centrifuged (10 min at 1600 *g*) to facilitate separation of the layers. The plasma fraction was frozen in a dry-ice—acetone bath and the organic layer transferred to a clean 15-ml screw-cap test tube. An aliquot (1 ml) of freshly prepared 7.5% aqueous ammonium hydroxide was added to the ethyl acetate, then shaken and centrifuged as above. The ethyl acetate was discarded by aspiration and the remaining aqueous portion acidified with 2 ml of 1 *M* sulfuric acid. Drug was then back-extracted into a single 5-ml volume of ethyl acetate and centrifuged as previously described. After freezing the acidic fraction, the organic layer was transferred to a 20-ml glass vial for evaporation under a stream of nitrogen in a water bath (45°C). The final residue was dissolved in 0.5 ml of the mobile phase and then automatically injected (0.1 ml) onto the HPLC column.

Detector response and calibration

A standard curve was generated in an attempt to bracket the range of HCT urinary concentrations anticipated over a 48 h period following the oral administration of 25 mg of HCT to human subjects. The linearity of detector response was initially investigated after repeated ($n = 6$) injections (0.1 ml) of standard solutions prepared by dilution with mobile phase to contain 0.5, 5, 10, 20, 30, 40 and 50 μg/ml HCT and a constant concentration (5 μg/ml) of the internal standard. Peak-area ratio (HCT/TCMT) versus HCT concentration data from the response curve were evaluated by least-squares fit analysis.

Selectivity

The chromatographic behavior of chlorothiazide, propranolol, pindolol, metoprolol, timolol and labetalol were evaluated to determine their potential for assay interference. In addition, drug-free human urine was routinely analyzed as described above and the resultant chromatograms examined for the presence of endogenous co-extractants which could interfere with the quantitation of either HCT or TCMT.

Drug recovery and inter-assay variability

Urine was collected from several unfasted human volunteers to generate a drug-free pool. The efficiency of extracting drug from urine was then determined using the following procedure. HCT and the internal standard were added to aliquots (1 ml) of urine (six replicates per group) to achieve HCT concentrations of 0.5, 10 and 50 μg/ml with a constant TCMT concentration of 5 μg/ml. Samples were then extracted as previously described and 0.1 ml of the reconstituted extract was automatically injected. In order to calibrate the detector response, extracts from each of three samples spiked to contain 10 μg/ml HCT were initially injected. Quantitation of HCT concentrations in the remaining samples was then automatically calculated by the computer using the average (n = 3) internal standard response factor generated at this calibration point. The recovery of HCT and TCMT was determined by comparing the peak area of both compounds from extracted samples to those obtained from the analysis of equivalent amounts of drug injected directly.

Stability of HCT

The stability of HCT and TCMT in the reagents used during the extraction procedure was examined. Solutions of both drugs were prepared (10 μg/ml) in 7.5% ammonium hydroxide, 0.1 *M* sodium bicarbonate and 1 *M* sulfuric acid, stored for 24 h at room temperature, and then assayed by HPLC. The stability of HCT in urine samples was also evaluated in the following manner. Aliquots (1 ml) of freshly collected drug-free urine (24 replicates per group) were spiked with HCT to achieve concentrations of 0.5, 10 and 50 μg/ml and then stored frozen (−20°C). At varying time intervals (0, 6, 13, 29 and 60 days), four samples from each concentration group and two aliquots of drug-free urine were assayed for HCT as described. The analytical results from each group were then evaluated to determine whether significant changes in concentration had occurred as a function of storage time.

RESULTS AND DISCUSSION

Chromatography

Baseline resolution was achieved between HCT (5.5 min) and TCMT (9.8 min) under the chromatographic conditions described. Optimization of chro-

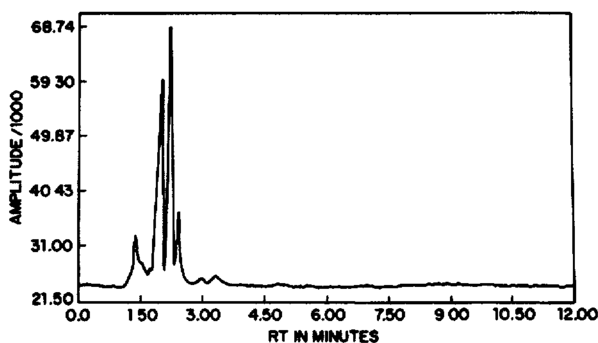

Fig. 1. Computer-reconstructed chromatogram of an extract from drug-free human urine.

matography to accommodate column-to-column variation in performance was accomplished by modest changes in the pH (6.95—7.25) of the mobile phase. In general, the retention of HCT was relatively insensitive to pH in this range, whereas a significant increase in the retention of TCMT was observed as the pH approached 6.95. Chlorothiazide, a structurally similar diuretic, was poorly retained (2.25 min) while labetalol remained undetectable at concentrations as high as 10 μg/ml. The retention times of timolol, pindolol, metoprolol and propranolol were 13.4, 14.0, 17.6 and 91 min, respectively. The presence of these compounds in urine demonstrated little or no potential for interference in this assay. Extracts from drug-free urine were found to be free of interfering peaks (Fig. 1). Representative chromatograms from urine spiked with 0.50 and 50 μg/ml of HCT are shown in Fig. 2.

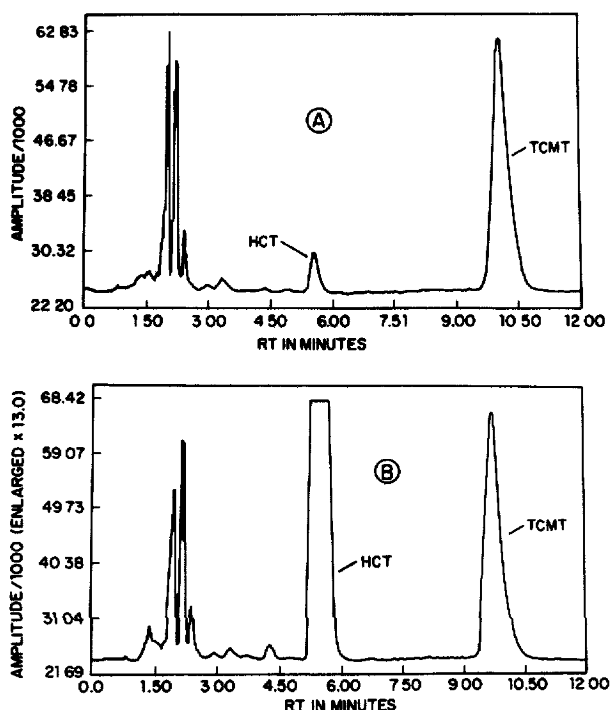

Fig. 2. Computer-reconstructed chromatograms of (A) an extract from urine spiked to contain 0.5 μg/ml HCT and (B) an extract from urine spiked to contain 50 μg/ml HCT. Both samples (1.0 ml) were fortified with internal standard (TCMT) to achieve a concentration of 5 μg/ml.

Detector response and calibration

The integrated peak area (μV-s) ratio of HCT to TCMT was chosen as the quantitative measure of detector response. Weighted (1/variance) regression analysis of these data indicated that the best-fit straight-line relationship between detector response (area ratio) and HCT concentration had a coefficient of determination (r^2) greater than 0.999. The slope was calculated to be 0.260 μg/ml and the y-intercept, which was not significantly different from zero at the 95% confidence interval, was determined to be 0.0015.

Drug recovery and intra-assay variability

The average recovery (± S.D.) of HCT from urine samples to which drug standards had been added at concentrations ranging from 0.50 to 50 μg/ml was determined to be 70.8 ± 3.08% (Table I). The internal standard (5 μg/ml) was extracted with a mean (± S.D.) efficiency equal to 81.9 ± 2.65%. Statistical analysis by single-level ANOVA demonstrated that there were no significant differences ($p > 0.05$) among the mean recoveries for HCT, nor were there significant differences in the recovery of TCMT as a function of HCT concentration. These data, therefore, suggest that there was no recovery dependence on concentration over the range of drug levels in urine which were investigated. Concentration data for urine, fortified to contain 0.5, 10 and 50 μg/ml, were automatically calculated by the integrating computer using the internal standard method from a single point calibration (10 μg/ml HCT and 5 μg/ml TCMT), and are shown in Table II. The estimates of drug concentration were found to be highly reproducible (mean coefficient of variation 4.52%) and ranged in relative accuracy from +2.82 to +8.37%. An additional group (n = 8) of urine samples were fortified to contain 0.25 μg/ml HCT and then analyzed to further challenge the lowest limit of reliable quantitation. The mean (± S.D.) concentration was determined to be 0.231 ± 0.009 μg/ml with a relative bias of −7.45%.

TABLE I

PERCENTAGE RECOVERY OF HCT AND THE INTERNAL STANDARD (TCMT) FROM HUMAN URINE AT VARYING HCT CONCENTRATIONS

n	HCT concentration (μg/ml)	Percentage recovery HCT (mean ± S.D.)	TCMT concentration (μg/ml)	Percentage recovery TCMT (mean ± S.D.)
6	0.5	69.47 ± 2.00	5.0	81.00 ± 2.70
6	10.0	69.73 ± 3.56	5.0	82.48 ± 1.70
5	50.0	73.57 ± 1.79	5.0	82.37 ± 3.65
17		70.77 ± 3.08		81.93 ± 2.65

TABLE II

INTRA-ASSAY PRECISION AND ACCURACY OF HCT ANALYSIS IN HUMAN URINE BY HPLC (n = 6)

Theoretical concentration (μg/ml)	Observed concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	Percentage bias
0.5	0.542 ± 0.0256	4.72	+8.37
10.0	10.28 ± 0.420	4.09	+2.82
50.0	52.41 ± 2.72	5.19	+4.82

Stability of HCT

No evidence for the hydrolytic decomposition of either HCT or TCMT stored at room temperature for 24 h in the reagents used for extraction was

TABLE III

ANALYTICAL RESULTS FROM TWO-MONTH STABILITY STUDY FOR URINE SPIKED TO CONTAIN 0.5, 10.0 AND 50.0 μg/ml HCT

Day No.	*n*	Theoretical concentration (μg/ml)	Mean observed concentration (μg/ml)	Coefficient of variation (%)	Percentage bias
0	4	0.500	0.486	1.47	−2.9
6	4	0.500	0.487	1.54	−2.66
13	4	0.500	0.494	2.16	−1.25
29	4	0.500	0.534	1.70	+6.8
60	4	0.500	0.504	1.54	−0.7
	20		0.501	3.96	+0.138
0	5	10.0	10.26	1.5	+2.63
6	4	10.0	9.84	2.8	−1.60
13	4	10.0	10.35	1.10	+3.48
29	4	10.0	10.69	1.57	+6.87
60	4	10.0	9.79	0.37	−2.09
	21		10.19	3.60	+1.89
0	4	50.0	46.56	1.15	−6.88
6	4	50.0	47.10	2.14	−5.80
13	4	50.0	46.56	1.62	−6.87
29	4	50.0	50.13	1.73	−0.26
60	4	50.0	45.76	0.42	−8.48
	20		47.22	3.57	−5.56

observed. The results from a two-month stability study of HCT in urine are shown in Table III. A statistical examination of the data generated on days 0, 6, 13, 29 and 60 revealed no significant ($p > 0.25$) change in HCT concentration with time.The design of this two-month stability study was also useful in providing additional estimates of inter- and intra-assay variability (Table III). These data confirm that the within-day and day-to-day precision of the method is well within acceptable limits over an extended concentration range of HCT in urine.

TABLE IV

URINARY EXCRETION OF UNCHANGED HCT FROM ONE SUBJECT TREATED (PER OS) WITH 25 mg OF HCT

Time (h)	HCT concentration (μg/ml)	Urine volume (ml)	Cumulative percentage dose
0—2	20.19	105	8.48
2—4	40.63	120	27.98
4—8	13.43	395	49.30
8—12	8.75	250	58.05
12—24	4.24	500	66.53
24—36	2.49	455	71.06
36—48	0.50	1040	73.14

Urinary excretion of HCT

Representative urinary excretion data from one of several subjects enrolled in a clinical bioavailability study and treated (per os) with 25 mg of HCT are shown in Table IV. Drug levels ranged from 0.5 to 40.6 μg/ml within a 48-h collection period. Conversion of these data to cumulative percent dose revealed that nearly 73% of the dose was excreted as unchanged drug in the urine. These findings are consistent with previously reported [2—7] results.

CONCLUSIONS

In summary, an HPLC method for the quantitative determination of HCT in human urine has been validated for concentrations ranging from 0.25 to 50 μg/ml. Selectivity for HCT in the presence of chlorothiazide, trichloromethiazide and a variety of β-blocking agents was demonstrated. The improved limit of reliable quantitation (0.25 μg/ml) has proven particularly useful in evaluating the HCT urinary excretion rate during the 36—48 h time interval. This assay is currently employed for the routine measurement of HCT in urine following the concomitant administration of HCT (25 mg) and labetalol.

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