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Fully automated methods for the determination of hydrochlorothiazide in human plasma and urine

J.Y.-K. HSIEH, C. LIN,* B.K. MATUSZEWSKI and M.R. DOBRINSKA

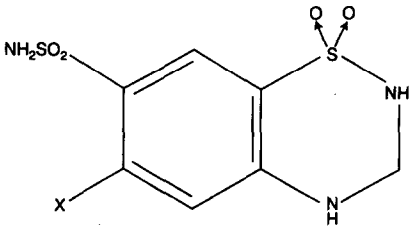
Department of Drug Metabolism, Merck Research Laboratories, West Point, PA 19486, USA

Abstract: LC assays utilizing fully automated sample preparation procedures on Zymark PyTechnology™ Robot and BenchMate™ Workstation for the quantification of hydrochlorothiazide (HCTZ) in human plasma and urine have been developed. After aliquoting plasma and urine samples, and adding internal standard (IS) manually, the robot executed buffer and organic solvent addition, liquid–liquid extraction, solvent evaporation and on-line LC injection steps for plasma samples, whereas, BenchMate™ performed buffer and organic solvent addition, liquid–liquid and solid-phase extractions, and on-line LC injection steps for urine samples. Chromatographic separations were carried out on Beckman Octyl Ultrasphere column using the mobile phase composed of 12% (v/v) acetonitrile and 88% of either an ion-pairing reagent (plasma) or 0.1% trifluoroacetic acid (urine). The eluent from the column was monitored with UV detector (271 nm). Peak heights for HCTZ and IS were automatically processed using a PE-Nelson ACCESS*CHROM laboratory automation system. The assays have been validated in the concentration range of 2–100 ng ml^{-1} in plasma and 0.1–20 μg ml^{-1} in urine. Both plasma and urine assays have the sensitivity and specificity necessary to determine plasma and urine concentrations of HCTZ from low dose (6.25/12.5 mg) administration of HCTZ to human subjects in the presence or absence of losartan.

Keywords: *Diuretic and antihypertensive agent; hydrochlorothiazide; liquid chromatography; laboratory robotics; PyTechnology™ robotic system; BenchMate™ workstation; automated sample preparation methods.*

Introduction

Hydrochlorothiazide,[6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide] (HCTZ, I, Fig. 1) is a diuretic and antihypertensive agent shown to be effective in the treatment of hypertension, either alone or in combination, to enhance the effectiveness of other antihypertensive drugs [1]. The bioavailability of HCTZ in human is 60–80% [2], and is independent of the dose over the 25–200 mg range [3]. After oral administration, peak plasma concentration occurs at 2 h and the half-life of elimination averages 10 h [4]. Urinary recovery is the preferred way of estimating bioavailability [4, 5] in humans because more than 95% of the intravenous dose is recovered unchanged in the urine [2]. To support HCTZ/losartan (12.5 mg:50 mg) clinical (bioequivalence/bioavailability/interaction) studies, a quantitative bioanalytical method was required which was both specific for HCTZ in the presence of losartan (a novel angiotensin II receptor antagonist) as well as sensitive enough to measure concentrations of HCTZ in plasma or urine following the low dose (6.25 and 12.5 mg) administration of HCTZ to human subjects.



I, X=Cl
II, X=Br

Figure 1
Chemical structures for HCTZ and internal standard.

Numerous analytical methods have been reported in the literature for the determination of HCTZ in biological fluids. These methods include colorimetry [6], thin-layer chromatography [7], gas–liquid chromatography [8, 9] and liquid chromatography (LC) [10–16]. Colorimetric assay lacks specificity and sensitivity. Gas–liquid and thin-layer chromatographic methods require tedious sample preparation, clean-up and derivatiz-

* Author to whom correspondence should be addressed.

ation procedures. LC methods described in the literature either require large volumes of plasma aliquots for extraction or utilize labour-intensive manual extraction procedures.

A more efficient and less labour-intensive means of preparing biological samples for analysis evolved with the emergence of laboratory robotics in the early 1980s. It has been shown that laboratory robotics are adaptable to performing steps such as liquid–liquid and liquid–solid extraction which are generally required to isolate drugs from the biological matrix and successfully inject samples into the LC system. Automated methods for the quantitation of several investigational drugs in human plasma have been developed, validated and implemented in our laboratories with equal or improved accuracy and precision than manual procedures, and increased sample throughput. In this report, we describe two validated methods comprised of Zymark robotic and BenchMate™ Workstation Systems interfaced to two LC systems with ultraviolet (UV) detection for quantifying HCTZ concentrations in human plasma and urine, and applications of these methods to support clinical pharmacokinetic studies. Both integrated systems provided unattended sample preparation and analysis, as well as automated retrieval and computation of the chromatographic results, and were capable of analysing over 100 samples per day.

Experimental

Materials

LC-grade acetonitrile and methanol, and reagent-grade sodium carbonate, sodium bicarbonate and sodium phosphate (monobasic) were purchased from Fisher (Pittsburgh, PA). High-purity methyl *tert*-butyl ether (MTBE) was obtained from Burdick and Jackson (Muskegon, MI, USA). Trifluoroacetic acid was supplied by the Sigma Chemical Co. (St Louis, MO, USA). Tetramethylammonium chloride was obtained from Fluka (St Louis, MO, USA). HCTZ and its bromo-analogue (IS, II, Fig. 1) were from Merck Research Laboratories (Rahway, NJ, USA). Heparinized human control plasma was obtained from Biological Speciality (Lansdale, PA, USA). Solid-phase extraction (SPE) was performed using silica-gel cartridge (50 mg, 3 ml) from Baker (Phillipsburg, NJ, USA).

Instrumentation and chromatographic conditions

A Zymark PyTechnology II™ robotic system (Hopkinton, MA, USA) was used to automate the plasma sample preparation and analysis. System peripherals included a SYSTEM V™ Controller, a general purpose hand, a pipetting hand G (1.0 ml), two 50-tube racks, a temperature controllable sample rack, a weighing station equipped with an analytical balance (Mettler AE240, Highstown, NJ, USA), a tube dispenser, a dilute and dissolve station, a liquid–liquid extraction station, an evaporation station with an in-house built fumehood, a centrifuge station, an LC sipping injector, a waste disposal station, two master laboratory stations, and two power and event controllers. A Haake (West Germany) refrigerated bath and circulator was used with the cooled sample rack station. The LC system included a Perkin–Elmer Series 10 Pump (Norwalk, CT, USA), a Kratos Model 773 absorbance detector (Ramsey, NJ, USA), a SSI column inlet filter (1.5 mm × 0.5 μm, State College, PA, USA), a Supelco Supelguard LC-8 column (2 cm × 2.5 mm, 5 μm, Bellefonte, PA), and a Beckman octyl ultrasphere analytical column (25 cm × 4.6 mm, 5 μm, Berkeley, CA, USA). Figure 2 shows the layout of the PyTechnology II™ robotic system and the on-line LC equipment. Chromatographic separations were performed using a mobile phase consisting of 980 ml of acetonitrile and 6000 ml of deionized water containing 16 g of tetramethylammonium chloride and 12 g of sodium phosphate (monobasic) at ambient temperature (pH 4.9) with a flow rate of 1.2 ml min^{-1}. The eluent was monitored with the UV detector set at the wavelength of 271 nm.

The automated sample preparation and chromatographic analysis of HCTZ in human urine was performed using an integrated Zymark BenchMate™ Workstation (basic workstation equipped with a solid-phase extraction and an on-line LC injector units), to which a Perkin–Elmer Series 250 pump and an Applied Biosystems (Ramsey, NJ, USA) model 785A programmable absorbance detector were linked. A mobile phase consisting of a mixture of 0.1% trifluoroacetic acid and acetonitrile (88:12, v/v) (pH* = 2.1) was delivered at a flow rate of 1.3 ml min^{-1} through the same chromatographic flow system (column inlet filter, guard and analytical

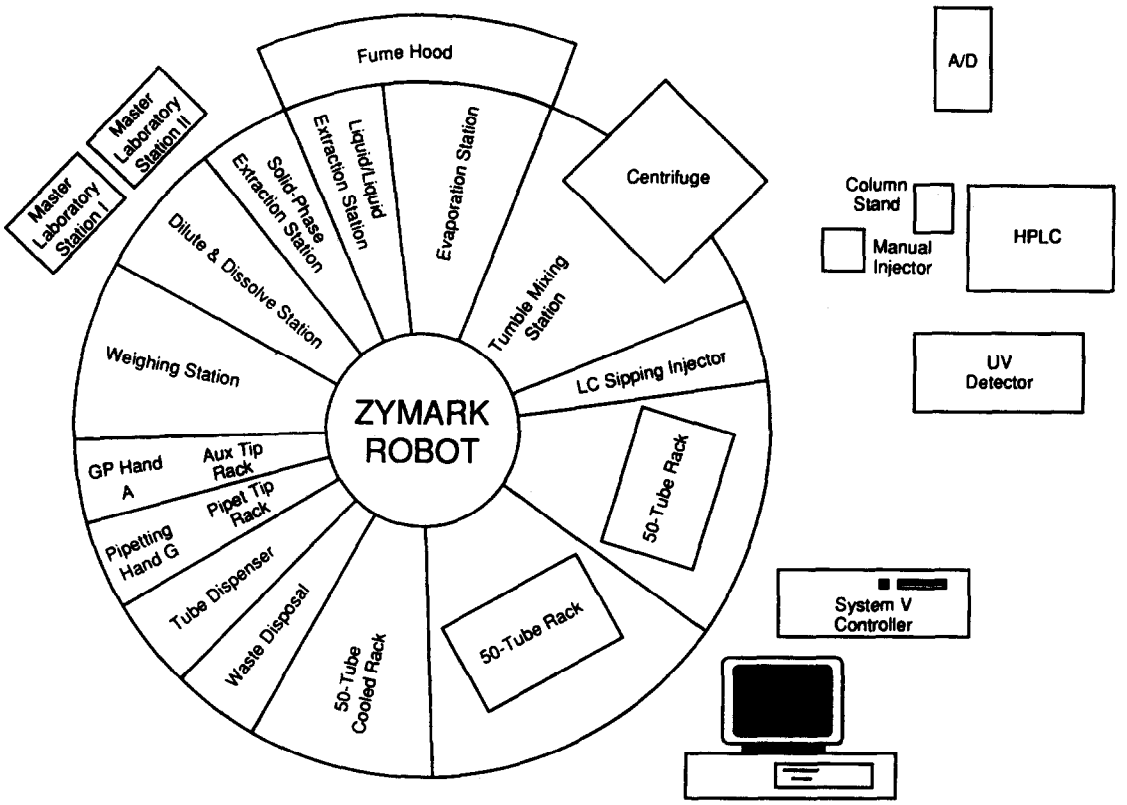


Figure 2
Layout of the PyTechnology™ robotic system and the on-line LC equipment.

columns) as utilized in the analysis of plasma. The wavelength of the UV detector was 271 nm and the loop size of the LC injector was 100 μl.

Preparation of standards

A stock solution of HCTZ (1 mg ml^{-1}) was prepared in methanol in amber glass scintillation vial. Appropriate dilutions of the stock were made with methanol and the working standard solutions were used to spike into 1 ml of control human plasma and urine at final concentrations of 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng ml^{-1}, and 0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 μg ml^{-1}, respectively. Plasma and urine quality control (QC) samples were prepared separately in pools of 200 ml at final concentrations of 3.75 and 75.0 ng ml^{-1}, and 0.75 and 15 μg ml^{-1}, respectively. All the QC samples were stored at −15°C until use. A stock solution of internal standard (IS) was prepared at 100 μg ml^{-1}, first diluted to 10 μg ml^{-1} with methanol, and further diluted to 1 μg ml^{-1} with a mixture of methanol and water (1:9, v/v). One hundred microlitres of the working IS solution was pipetted into calibration standard and unknown subject plasma samples. Fifty microlitres of the stock IS solution was used for all the urine samples.

Sample preparation

Plasma. A 0.5–1.0-ml aliquot of human plasma, 100 μl of methanol, and 100 μl of working internal standard solution (1 μg ml^{-1}) were pipetted into a 16 × 100 mm glass culture tube containing 0.5–1.0 ml of make-up control plasma. The tube was vortex-mixed for 30 s on a vortex-mixer and then loaded onto a thermostated (5°C) rack of a Zymark Py Technology™ System (Fig. 2). The robot executed a number of steps including buffer and organic solvent addition, liquid–liquid extraction of the extract to dryness, evaporation and on-line HPLC injection as outlined in Sequence 1.

Sequence 1. Automated steps performed by the Zymark Py Technology™ robotic system during analyses of HCTZ in human plasma were as follows:

add 0.5 ml of 0.1 M $NaHCO_3$ (pH 9.0) and 5 ml of MTBE;

vortex for 150 s at a speed of 65 cycles/s to

extract HCTZ and IS into the organic phase;
centrifuge for 12 min at 2000*g*;
transfer 4.5 ml of organic phase into another tube;
evaporate the organic phase to dryness under a stream of N_2 at 40°C;
reconstitute the residue in 0.25 ml mixture of acetonitrile–water (1:4, v/v), and vortex-mix for 1 min; and
inject the sample (100 μl) into LC system.

Urine. Subjects' urine samples were thawed to room temperature and vortex-mixed vigorously for 30 s on a vortex-mixer. A 1 ml aliquot of urine and 50 μl of stock internal standard solution (100 μg ml^{-1}) were pipetted into a disposable culture glass tube (16 × 100 mm). The Zymark BenchMate™ Workstation executed liquid–liquid, solid-phase extraction, and on-line LC injection steps as described in Sequence 2.

Sequence 2. Automated steps performed by BenchMate™ Workstation during the assay of HCTZ in human urine were as follows:
add 0.5 ml of 0.1 M $NaHCO_3$ buffer (pH = 9.4);
add 4 ml of MTBE;
vortex for 120 s at speed 3;
condition SPE cartridge with 2 ml of methanol;
condition cartridge with 3 ml of MTBE;
load 3.8 ml of MTBE extract onto the SPE silica cartridge;
dry cartridge with nitrogen gas for 60 s;
wash robotic syringe with 2 ml of aqueous methanol (20%);
elute HCTZ and IS from cartridge using 2 ml aqueous methanol (20%);
inject 50 μl of the eluent on the LC system (run time of 12 min); and
wash sample loop with 2 ml of aqueous methanol (20%).

Data acquisition and analysis

Peak heights for HCTZ and IS were acquired and automatically processed using a PE-Nelson ACCESS*CHROM laboratory automation system. HCTZ concentrations in study samples were calculated from the daily least-squares linear regression of peak height ratios vs standard amounts, with reciprocal weighing on the amounts. When a calculated amount exceeded the standard curve range, the sample was diluted and reanalysed.

Results and Discussion

System characteristics

To increase the number of plasma samples analysed per day, the robot was programmed through the use of Easylab™ software to operate in a scheduled fashion. Each sample was moved through the stations in a serial fashion rather than as a batch of samples being processed together from step to step which is what is done manually. To utilize all of the robot's time, several of these serial samples were interleaved as they were assayed such that while the robot was waiting for one sample to complete a long non-robotic step (such as evaporation) the robot can process another sample. Typically, it took the robot 26 h to analyse 100 plasma samples.

The laboratory unit operations (LUO) such as liquid dispensing and aspirating that were used in plasma assay have been tested and validated by a weighing procedure. The validation data for several LUOs are shown in Table 1, indicating excellent precision of all operations. Similar validation data for several BenchMate™ operations used in urine assay are shown in Table 2 confirming the aceptable precision of all steps employed. Using the BenchMate™ system about 100 urine samples can be analysed over a 24-h period.

Initially, an attempt was made to perform the assay in plasma under the same LC conditions as utilized for the assay in urine. However, a number of impurities extracted from plasma co-eluted with HCTZ and IS, and

Table 1
Validation of the laboratory unit operations

Laboratory unit operation	% RSD*
Dispense 0.5 ml 0.1 M $NaHCO_3$	0.8
Dispense 5 ml methyl *t*-butyl ether	0.02
Aspirate and dispense 4.5 ml methyl *t*-butyl ether	0.9
Dispense 0.25 ml mobile phase	1.0

*$n = 6$.

Table 2
Validation of BenchMate™ workstation operations

BenchMate™ workstation operation	% RSD*
Dispense 4 ml methyl *t*-butyl ether	0.3
Dispense 0.5 ml H_2O	1.9
Aspirate and dispense 4 ml methyl *t*-butyl ether	0.9
Collect 2 × 1 methanol fractions	1.4

*$n = 5$.

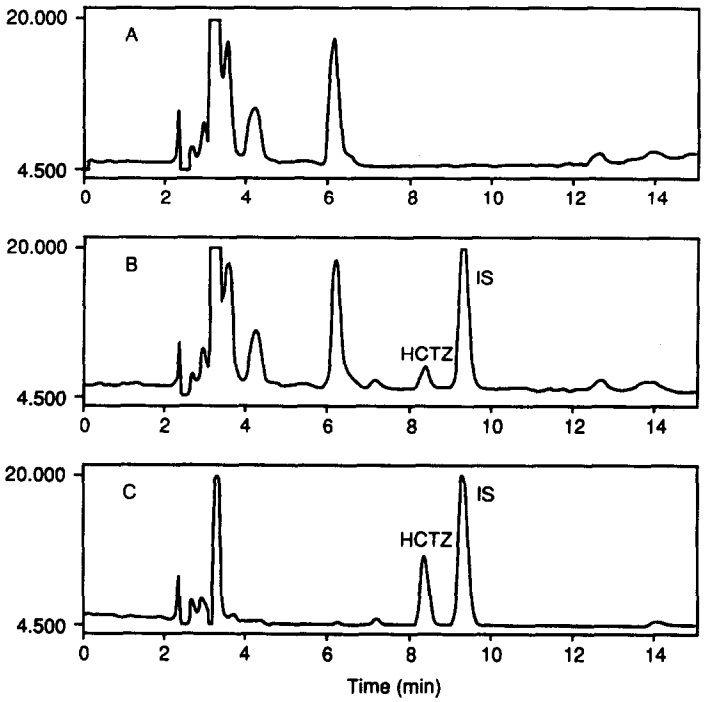


Figure 3
Representative chromatograms of hydrochlorothiazide (HCTZ) and internal standard (IS) in human plasma (1 ml). (A) Control plasma blank, (B) control plasma spiked with 5 ng ml^{-1} of HCTZ and 50 ng ml^{-1} of IS, (C) 0.5-h plasma samples from subject no. 9 given a Merck 50-mg losartan tablet and a 12.5-mg HCTZ tablet (18.3 ng ml^{-1} of HCTZ and 50 ng ml^{-1} of IS).

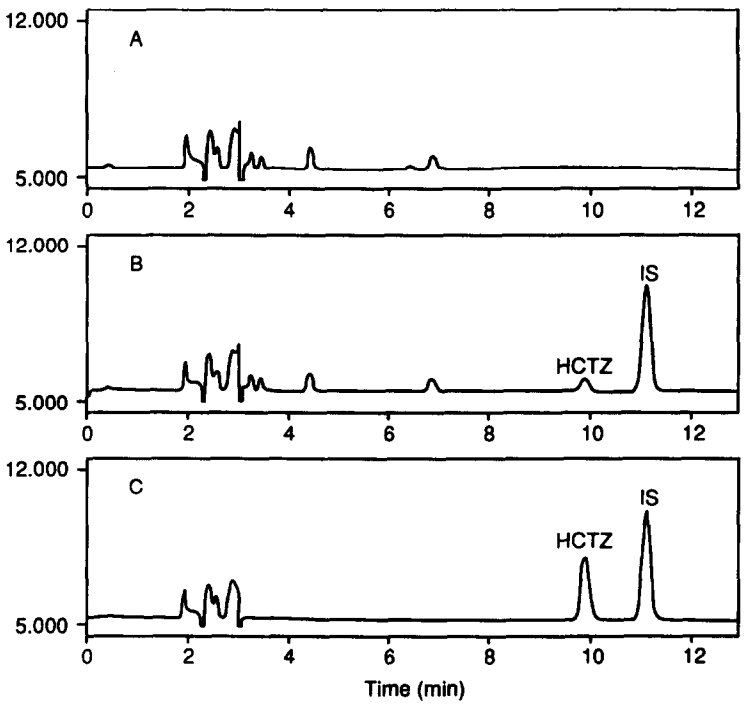


Figure 4
Representative chromatograms of hydrochlorothiazide (HCTZ) and internal standard (IS) in human urine (1 ml). (A) Control urine blank; (B) control urine spiked with 0.5 μg ml^{-1} of HCTZ and 5 μg ml^{-1} of IS; (C) 4–6-h urine sample from subject no. 13 given a Merck 50-mg losartan tablet and a 12.5-mg HCTZ tablet (2.32 μg ml^{-1} of HCTZ and 5 μg ml^{-1} of IS).

Table 3
Reproducibility of daily calibration curves

	Slope (m)		Intercept (I)		Correlation coefficient (r^2)	
	Plasma*	Urine†	Plasma*	Urine†	Plasma*	Urine†
Mean	0.0249	0.2507	0.0071	0.0022	0.9986	0.9999
SD (±)	0.0012	0.0133	0.0046	0.0022	0.0022	0.0003
% RSD	4.7	5.3	—	—	0.2	0.03

*n = 14.
†n = 9.

interfered with their quantitation. Therefore, for the assay in plasma, the LC conditions were changed, and the mobile phase utilized in urine assay was replaced with a mobile phase containing acetonitrile and ion-pairing reagent (tetramethylammonium chloride). Under these ion-pairing conditions, all endogenous impurities from plasma were separated from both HCTZ and IS.

Assay validation

Typical chromatograms of control human plasma and urine, and plasma and urine spiked with HCTZ (5 ng ml^{-1} and 0.5 μg ml^{-1}, respectively) and internal standard are shown in Figs 3 and 4. HCTZ and internal standard were eluted with retention times of 8.4 and 9.2 min in plasma, and 9.9 and 10.9 min in urine, respectively. The specificity of the assays was illustrated by the lack of interference observed at the retention times of these compounds in any of the control and predose plasma and urine samples tested. In addition, under the LC conditions utilized, losartan and its metabolite were retained on the LC column, and were not interfering with the determination of HCTZ. After each daily run, the analytical column was washed with a mixture of water and methanol (20:80, v/v) to remove losartan and its metabolite accumulated on the column.

Linearity of the calibration curve was established in the range of 2–100 ng ml^{-1} in plasma and 0.1–20 μg ml^{-1} in urine. Samples with concentrations above the linear calibration range were diluted and assayed again. Standard curves of HCTZ in plasma and urine were constructed by plotting peak height ratio (HCTZ/IS) vs drug concentration. The calibration curve was fitted using the $Y = mX + I$ equation and weighted by $1/Y$ (where m is slope and I is intercept). Calibration curve data are shown in Table 3. An average correlation factor (r^2) of 0.9986 for plasma and 0.9998 for urine was obtained. Mean slope data were associated with coefficient variations of 4.7 and 5.3%, respectively, indicating good between-day assay reproducibility.

Prior to the analysis of clinical samples, various amounts of HCTZ for constructing calibration curves and fixed amount of IS were added to control plasma and urine, and five replicate samples were assayed to assess intra-day variability. The means standard deviations (SD) and percentage relative standard deviations (% RSD) were computed at each concentration. Table 4 provides intraday precision data for the analyses of HCTZ in plasma and urine. All % RSD values were <10%. The interday variability was determined by analysing quality control samples (QC) prepared at high and low concentrations within the calibration curves and stored at −15°C. Inter-day precision was below 4.0% in plasma and urine (Table 5). The accuracy data (percentage deviation from nominal values) based on QC standard at 3.75 and 75 ng ml^{-1} in plasma, and at 0.75 and 15 μg ml^{-1} in urine, were −1.3 to −1.2%, and +4.0 to +0.9%,

Table 4
Intraday precision data for the analysis of HCTZ in human plasma and urine

Plasma		Urine	
Concentration (ng ml^{-1})	% RSD	Concentration (μg ml^{-1})	% RSD
2.0	5.0	0.10	8.4
5.0	2.2	0.50	3.7
10.0	3.1	1.00	0.9
20.0	1.2	5.00	0.6
50.0	0.9	10.00	1.3
100.0	1.0	20.00	0.8
3.75 (LQC)*	4.7	0.75 (LQC)	1.5
75.0 (HQC)†	1.2	15.0 (HQC)	0.6

*LOQ = Low quality control.
†HQC = High quality control.

Table 5
Interday variability for the analysis of quality control plasma and urine samples spiked with HCTZ

Nominal concentration	Mean concentration	*n*	% RSD (% deviation from nominal)
Plasma			
3.75 ng ml^{-1}	3.70 ng ml^{-1}	19	3.2 (−1.3)
750.0 ng ml^{-1}	74.1 ng ml^{-1}	19	2.3 (−1.2)
Urine			
0.75 μg ml^{-1}	0.78 μg ml^{-1}	14	1.8 (±4.0)
15.0 μg ml^{-1}	15.1 μg ml^{-1}	14	3.9 (±0.9)

respectively. The data in Table 5 also indicate the excellent stability of HCTZ in plasma and urine during storage at −15°C for at least 3 months.

Extraction efficiencies of HCTZ in plasma and urine were determined by comparing peak height of the analytes from extracted plasma and urine to those of directly injected standards. Across the range of the calibration curve, the recovery of HCTZ ranged from 83 to 90% in plasma and from 20 to 25% in urine. The low recovery in urine could be attributed either to poor efficiency of the liquid–liquid extraction step or a loss of the drug during SPE on a silica cartridge. In order to differentiate between these two possibilities, HCTZ was spiked directly into MTBE (4 ml), the solution was applied to the cartridge and treated the same way as during the assay in urine. Recovery of HCTZ from the cartridge was practically 100%, indicating the poor recovery from urine was due to the loss of the drug during the liquid–liquid extraction step.

The efficiency of the liquid–liquid extraction of HCTZ from urine was pH dependent. The pK_a values for HCTZ are 7.9 and 9.2, and the extraction efficiency in urine at pH 9.4 was lower than at pH 9.0 used in the plasma assay. When extraction from urine at pH 9.0 was attempted, recovery of the drug was higher than at pH 9.4, but a number of endogenous urine impurities were extracted and interfered with the integration of HCTZ and IS peaks. The final pH chosen was based on a compromise between adequate drug recovery and improved assay specificity. The recovery of HCTZ from urine at pH 9.4 was constant over the concentration range studied allowing reliable quantitation of the analyte. In addition, the sensitivity of the assay in urine below 100 ng ml^{-1} was not required, and lower recovery had no effect on HCTZ quantitation at these levels.

The limit of quantification [defined as the lowest concentration on the calibration curve with acceptable accuracy and precision (% RSD <10%)] were 2.0 ng ml^{-1} in plasma and 0.1 μg ml^{-1} in urine.

Analysis of clinical samples

The applicability of the developed method was demonstrated by analysing plasma and urine samples from three clinical studies. The mean plasma concentration vs time profiles obtained from 16 normal subjects following co-administration of 50-mg losartan and 12.5-mg HCTZ tablets and a DuPont–Merck 50-mg losartan/12.5-mg HCTZ tablet are shown in Fig. 5. The corresponding urinary recoveries (0–48 h) were 68.9 and 67.1%, respectively.

Conclusions

LC assays utilizing fully automated sample preparation procedures on Zymark PyTechnology II^{TM} robotic and BenchMate^{TM} Workstation systems have been developed and validated for quantifying HCTZ concentration

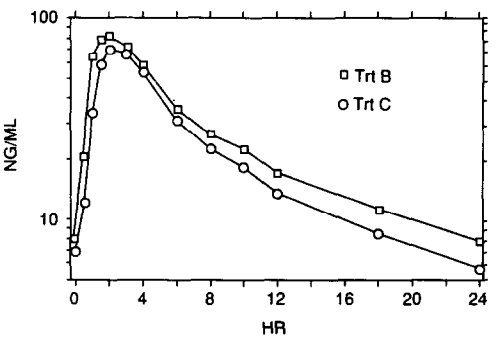


Figure 5
Mean plasma concentrations (ng ml^{-1}) of HCTZ (□-- Trt B, ○-- Trt C) following coadministration of 50-mg losartan and 12.5-mg HCTZ Tablets (Trt B) and as a DuPont-Merck 50-mg losartan/12.5-mg HCTZ Tablet (Trt C).

in human plasma and urine originating from bioequivalence/interaction studies. Both plasma and urine methods have been successfully applied to the determination of plasma and urine samples from normal subjects receiving oral doses of 6.25/12.5 mg HCTZ. Approximately 3500 plasma and urine samples, including calibration curve, quality control and clinical samples were assayed in less than 3 months of continuous operations using the two systems. The automation of the procedure led to the improvement in assay precision with similar accuracy and sensitivity as the manual methods but allowed unattended 24-h a day analyses of more than 100 clinical samples.

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