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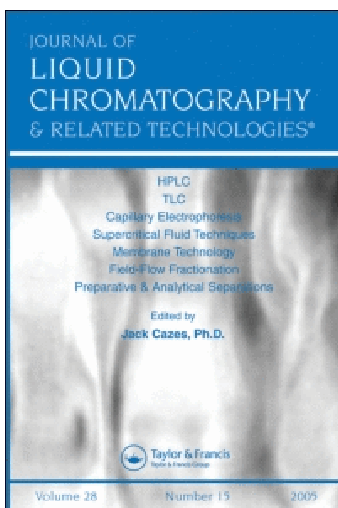

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W. Fang[a]; W. Xie[a]; J. Y. -K. Hsieh[a]; B. K. Matuszewski[a]
[a] Department of Drug Metabolism, Merck Research Laboratories, West Point, Pennsylvania, USA

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Development and Application of HPLC Methods with Tandem Mass Spectrometric Detection for the Determination of Hydrochlorothiazide in Human Plasma and Urine Using 96-Well Liquid-Liquid Extraction

W. Fang, W. Xie, J. Y.-K. Hsieh, and B. K. Matuszewski
Department of Drug Metabolism, Merck Research Laboratories,
West Point, Pennsylvania, USA

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Abstract: Sensitive and selective methods for the determination of hydrochlorothiazide (HCTZ) in human plasma and urine were developed. Both methods were based on high performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection. HCTZ and hydroflumethiazide (HFTZ), used as an internal standard, were isolated from human plasma and urine by liquid-liquid extraction in the 96-well plate format using a Tomtec Quadra 96 workstation. The MS/MS detection was performed on an Applied Biosystems-Sciex API 3000 tandem mass spectrometer interfaced with a heated nebulizer probe and operated in the negative ionization mode. The developed methods were validated in the concentration ranges of 1–100 ng/mL and 0.05–20 μg/mL in human plasma and urine, respectively. The precision of both methods, as expressed by the coefficients of variation (C.V.), was less than 5.6% at all concentrations within the standard curves with acceptable accuracy. The plasma and urine methods provided sufficient sensitivity and high sample throughput to map out the pharmacokinetics of HCTZ in human subjects following an oral dose of HCTZ/Losartan, in support of a human clinical trial designed to evaluate the bioequivalence of HCTZ between two formulations.

Keywords: HPLC-MS/MS determination, Hydrochlorothiazide, Hydroflumethiazide, 96-Well liquid-liquid extraction, Human plasma, Urine

Address correspondence to W. Fang, Department of Drug Metabolism, Merck Research Laboratories, WP26-372, Sumneytown Pike, West Point, PA 19486, USA. E-mail: wei_fang@merck.com

INTRODUCTION

Hydrochlorothiazide, 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfon-amide-1,1-dioxide (HCTZ, Fig. 1) is a thiazide diuretic that has been widely used for more than 40 years for the treatment of essential hypertension. It has been proven efficacious in controlling blood pressure. Thiazide diuretics work by affecting the renal tubular mechanisms of electrolyte reabsorption, directly increasing excretion of sodium and chloride. HCTZ can be used either alone or in combination with other drugs, such as Losartan (Fig. 1), a nonpeptide angiotensin II receptor antagonist that is active when administered orally. The combination of two antihypertensive agents can treat some hypertension patients more successfully than each single agent, with the potential advantage of a better side effect profile.

Bioanalytical methods described for the determination of HCTZ in human and animal biological fluids involved HPLC methods coupled to ultraviolet (UV),[1–13] or mass spectrometric detection.[14,15] Generally, HPLC methods with UV detection did not provide the required sensitivity (1 ng/mL or lower) and selectivity for clinical plasma analyses. In most HPLC-UV detection methods, large sample volume (0.5–1 mL) and lengthy sample clean up procedures were required to achieve the sensitivity of low nanograms per milliliter. To improve assay efficiency and reduce labor intensity, Hsieh et al. published fully automated HPLC-UV methods[13] for

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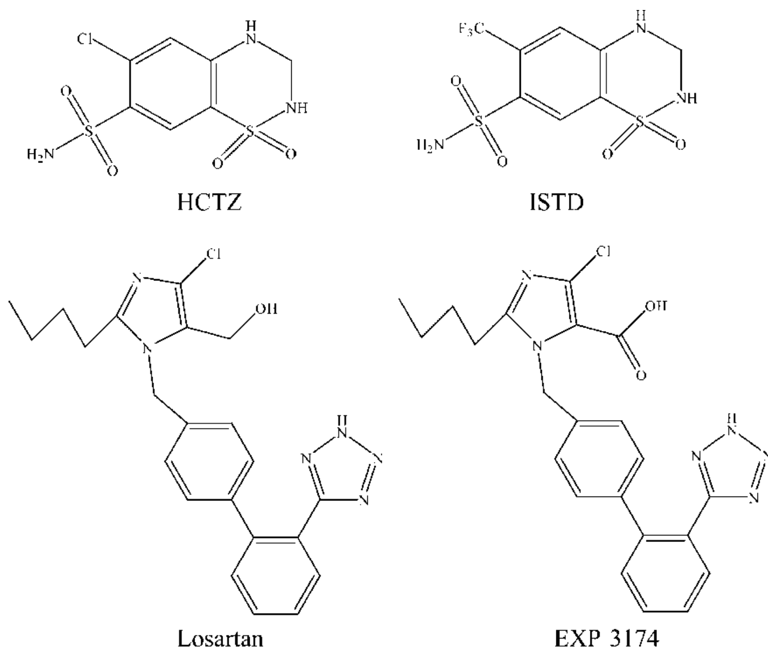

Figure 1. Chemical structures of HCTZ, ISTD, Losartan, and EXP-3174.

the determination of HCTZ using a Py Technology system and a Zymark BenchMate workstation for liquid-liquid extraction (LLE) of HCTZ, and an internal standard from human plasma and urine, respectively. In their HPLC-UV method, 1 mL of human plasma was needed to achieve the lower limit of quantitation (LLOQ) of 2 ng/mL. The LLE technique is widely used for biological sample preparation and is generally considered to be adequate to cleanly isolate drugs and metabolites from biological matrices. However, conventional LLE is performed manually using individual test tubes, which is time-consuming and tedious. Zymark and Py Technology BenchMate systems provided more efficient and less labor-intensive means of preparing biological samples than manual operations, with similar accuracy and precision. However, the cost of both systems was rather high and samples were prepared in a serial fashion with a HPLC run time of 15 minutes per sample.

In comparison to UV detection, tandem mass spectrometric detection provides high sensitivity and selectivity for quantifying drug concentrations in biological samples. Because of the selectivity of MS/MS detection, the chromatographic separation of the drug from endogenous interfering substances became a less critical requirement in comparison with HPLC methods with UV detection, and the analysis time could be shortened. Furthermore, the high sensitivity of MS/MS detection has allowed using less volume of the biofluids to achieve the same LLOQ.

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Foltea et al. reported a HPLC-MS/MS method for the determination of HCTZ in human plasma by LLE with a LLOQ of 2 ng/mL.[14] Using MS/MS detection in a negative ionization mode, their run time was only 1.2 minutes and sample volume required was 0.5 mL. However, the time-consuming LLE sample preparation procedure was not automated and was performed in a manual mode. In addition, matrix effect on quantification was not evaluated.

Recently, while our work was in progress, Takubo et al. published a HPLC-electrospray ionization (ESI) tandem mass spectrometric method for determination of HCTZ in rat plasma.[15] In their study, a post-column acetonitrile addition technique was required to increase the MS/MS detector responses of HCTZ by 500 fold to achieve the LLOQ of 4 ng/mL in rat plasma. The precision of the method at LLOQs was relatively high (16.4%) and the ESI interface that is known to have the disadvantage of exhibiting matrix effect,[16] was utilized. Both HCTZ and HFTZ, used as an internal standard, were also manually extracted and the HPLC run time using a gradient elution was 12 minutes.

In recent years, Tomtec Quadra 96 workstation, a relatively lower cost robot for parallel sample preparation, has been widely accepted in the bioanalytical chemistry field of the pharmaceutical industry. Semi-automated LLE methods using a Tomtec Quadra 96 workstation combined with HPLC-MS/MS for the quantitation of several investigational drugs have been successfully developed, validated, and implemented in our laboratories with equal or improved accuracy and precision as manual procedures.[17–20] These

semi-automated extraction methods performed by a Tomtec Quadra 96 work-station allowed preparation of 96 samples at a time, which greatly simplified the sample preparation procedure and provided a high throughput support for sample preparation and analysis.

To support a crossover clinical study evaluating the bioequivalence after administration of a HYZAAR^{TM} (a 100 mg Losartan/12.5 mg HCTZ) tablet and the co-administration of a COZAAR^{TM} (100 mg Losartan) tablet and a HCTZ(12.5 mg) capsule, more sensitive and high throughput bioanalytical methods were required to determine concentrations of HCTZ in plasma and urine. In the method described in this paper, the semi-automated liquid-liquid extraction procedure in the 96-well plate format using a Tomtec Quadra 96 workstation was utilized and HPLC run time under isocratic elution condition was 6 minutes. The sensitive assays in both human plasma (LLOQ 1 ng/mL) and urine (0.05 μg/mL) were developed and the matrix effect using APCI interface, instead of ESI, was extensively studied and its absence was demonstrated. The precision of the assay in plasma and urine was excellent and did not exceed 5% in both matrices at all concentrations studied. In addition, the selectivity of both assays was demonstrated in the presence of the co-administrated Losartan and its major carboxylic acid metabolite.

The details of the development, validation, and application of sensitive and high throughput HPLC-MS/MS methods for the determination of HCTZ in human plasma and urine using a semi-automated 96-well LLE method are the subjects of this publication.

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EXPERIMENTAL

Materials

HCTZ, an USP standard with purity of 97%, was used as the drug reference standard. Hydroflumethiazide with purity of 96% was used as the internal standard (ISTD). Both standards were obtained from Sigma (Milwaukee, WI, USA). Losartan (2-*n*-butyl-4-chloro-1-[*p*-(*o*-1H-tetrazol-5-ylphenyl)ben-zyl]-imidazole-5-methanol monopotassium salt) and its acid metabolite EXP3174 (2-*n*-butyl-4-chloro-1-[2-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-imidazole-5-car-boxyl acid) were obtained from Merck Research Laboratories (Rahway, NJ, USA). The chemical structures of all the compounds are shown in Fig. 1. Acetonitrile (ACN) and methyl *t*-butyl ether (MTBE), both in Optima grade, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate (HPLC grade) purchased from J.T. Baker (Phillips-burg, NJ, USA), sodium carbonate and sodium bicarbonate (anhydrous) obtained from Fisher Scientific, were used as received. Deionized water was obtained by passing in-house water through a Millipore Milli-Q plus system (Bedford, MA, USA). Nitrogen (99.999%) was supplied by West Point

Cryogenics (West Point, PA, USA). 96-Well collection plates (1.2 & 2.4 mL) and mats were purchased from Marsh Biomedical (Rochester, NY, USA). Different lots of drug free human heparinized plasma were obtained from Biological Specialties Corp. (Colmar, PA, USA). Different lots of drug-free human urine were provided from in-house healthy male volunteers and all were stored at −20°C before use.

Mass Spectrometry

An Applied Biosystems-Sciex API 3000 triple quadruple mass spectrometer (Foster City, CA, USA) equipped with a heated nebulizer source operating in the negative ionization mode was used for the studies described herein. Multiple reaction monitoring (MRM) mode was utilized for quantitation. The heated nebulizer probe temperature was maintained at 450°C, and the nebulizing gas (air) pressure was set at 75 psi. The settings for the nebulizer, curtain, and collision gases were 9, 9, and 12 on the API 3000 mass spectrometer, respectively. All the source and instrument parameters for monitoring HCTZ, ISTD, Losartan, and EXP-3174 were optimized by infusion of standard solutions of 1,000 ng/mL each at a flow rate of 10 μL/min into the mobile phase pumped at 400 μL/min using a Perkin Elmer Series 200 pump (Norwalk, CT, USA).

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Preparation of Standard Solutions

Plasma Method

A stock solution of HCTZ (100 μg/mL) was prepared in ACN. The stock solution of HCTZ was further diluted with 50% ACN in water (v/v) to give a series of working standards with concentrations of 10, 25, 50, 100, 250, 500, and 1000 ng/mL. A stock solution (1 mg/mL) of ISTD was also prepared in ACN. A working standard solution of 500 ng/mL of ISTD, prepared by diluting stock solution with 50% ACN in water (v/v), was used for plasma samples analyses. All the standard solutions were stored at 4°C. Plasma standards were prepared by adding 40 μL of each working standard to 400 μL of human control plasma. The resulting plasma standard concentrations ranged from 1 to 100 ng/mL.

A stock solution for quality control (QC) samples was prepared separately by the same procedure using a separate weighing. QC samples were prepared by diluting the QC working solutions with human control plasma. QC samples at three concentrations (LQC (2 ng/mL); MQC (40 ng/mL); HQC (80 ng/mL)) were used to evaluate assay precision and accuracy. All QC samples were divided into 1 mL aliquots in separate cryo tubes and stored at −20°C until analysis.

Urine Method

A stock solution of HCTZ (1 mg/mL) was prepared in ACN. The stock solution of HCTZ was further diluted with 50% ACN in water (v/v) to give a series of working standards with concentrations of 0.1, 0.4, 1, 2, 4, 10, 20, and 40 μg/mL. A stock solution (1 mg/mL) of ISTD was also prepared in ACN. A working standard solution of 20 μg/mL of ISTD, prepared by diluting stock solution with 50% ACN in water (v/v), was used for urine samples analyses. All standard solutions were stored at 4°C. Urine standards were prepared by adding 50 μL of each working standard to 100 μL of human control urine. The resulting urine standard concentrations ranged from 0.05 to 20 μg/mL.

A stock solution for QC samples in human urine was prepared separately by the same procedure using a separate weighing. QC samples were prepared by diluting the QC working solutions with human control urine. QC samples at three concentrations (LQC (0.1 μg/mL); MQC (9 μg/mL); HQC (18 μg/mL)) were used to evaluate assay precision and accuracy. All QC samples were divided into 1 mL aliquots in separate cryo tubes and stored at −20°C until analysis.

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Chromatographic Systems

A Perkin-Elmer (Norwalk, CT, USA) binary 250 pump and a Varian (Wakefield, RI, USA) ProStar 96-well plate autosampler were used in this work. The separation of analytes was performed on an Ultra Phenyl column (100 × 3.2 mm, 3 μm) from Restek (Bellefonte, PA, USA) protected by an Ultra Phenyl guard column (10 × 4 mm, 3 μm) and a 0.5 μm in-line filter. Mobile phase, a mixture of ACN: 10 mM ammonium acetate (40 : 60, v/v), was delivered at a flow rate of 0.5 min/mL. The total run time was 6 minutes. Washing solution made of 70% ACN in water (v/v) was used for needle and flow path cleaning of the autosampler.

Sample Preparation and Extraction

Plasma Method

QCs and subject plasma samples were thawed at room temperature. After vortex-mixing (15 seconds) and centrifuging (×3,101 g) plasma samples for 10 minutes, 400 μL of plasma samples were added individually into a 2 mL deep 96-well plate. Forty microliters of 50% of ACN in water (v/v) was added to blank and QC/subject samples, followed by pipetting 40 μL of ISTD solution to each well of the plate, except the wells designated as the double blank plasma samples. The plate was then placed onto a Tomtec

Quadra 96 workstation for 96-well liquid-liquid extraction. Fifty microliters of 0.1 M sodium carbonate buffer solution (pH = 9.5) was added into each well of the plate by a Tomtec Quadra 96 workstation. After shaking the plate for 30 seconds, 1.14 mL of MTBE was added to each well using a Tomtec Quadra 96 workstation. The 2.4 mL deep 96-well plate was then sealed with a mat made of molded PTFE/silicone liner and roto-mixed for 20 minutes. After centrifugation for 5 minutes at $\times$1,449 g, 850 μL of the top organic layer was transferred into a 1.2 mL 96-well plate using a Tomtec Quadra 96 workstation. The organic extract was evaporated to dryness under heated N_2 stream. The residue was reconstituted in 150 μL of ACN:10 mM ammonium acetate (50 : 50, v/v). The plate was sealed with a cap mat followed by vortex-mixing for 1 minute and centrifuged at $\times$1449 g for 5 minutes. Twenty microliters of the reconstituted sample was injected into the HPLC-MS/MS system.

Urine Method

Urine sample preparation procedure was similar to plasma sample preparation procedure, except the following changes were made. One hundred micro liter of human urine sample, 50 μL of ISTD (10 μg/mL) solution, 1 mL of MTBE extraction solution, 300 μL of top MTBE layer, 350 μL reconstitution solution, and 10 μL injection volume were used instead of the respected values listed in the plasma method.

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Quantification

Data acquisition and quantification were performed using Sciex Analyst Software 1.1. Weighted (weighting factor = $1/x^2$ where x = standard concentration) least squares regression calibration curves were constructed by plotting the peak area ratios of analyte to ISTD *versus* standard concentrations. The standard curve was constructed daily. Plasma or urine samples spiked with standards were assayed along with QC and unknown plasma or urine samples.

RESULTS AND DISCUSSION

Optimization of Chromatographic and MS/MS Conditions

Prior to assay development, Q1 spectra in the positive and negative ionization modes were obtained by infusing HCTZ to a Sciex API 3000 triple quadruple mass spectrometer using a heated nebulizer interface. Since no signal was detected at the protonated molecular ion at $m/z = 298$ in the positive ionization mode, the negative ionization mode was chosen.

In the negative ionization mode, the abundant deprotonated molecular ions $[M-H]^-$ at m/z 296 and 330 of HCTZ and ISTD, respectively, were

present and chosen as the precursor ions in the MS/MS optimization experiments. In addition to the *m/z* 296 and 330 deprotonated molecules, both compounds show similar pairs of fragment ions (*m/z* 269/205 and 303/239), which are not connected with the presence of the Cl and CF_3 substituents on the aromatic moieties of HCTZ and ISTD, respectively (Fig. 2). These fragments, corresponding to the loss of 27 and 64 amu, originated probably from the loss of HCN and SO_2 molecules from HCTZ and ISTD. The proposed fragmentation mechanism, similar to the mechanism reported earlier by Popot et al.,[21] is shown in Fig. 3. The precursor → product ion pairs of *m/z* 296 → 269 and *m/z* 330 → 239 were chosen for monitoring HCTZ and ISTD in MRM mode, respectively.

Because Losartan was co-administrated with HCTZ at 100 mg dose in this bioequivalence study, it was necessary to avoid the cross-talk interference (discussed in the selectivity section below) between HCTZ, ISTD, Losartan, and EXP-3174. One way of avoiding this cross-talk interference is to chromatographically separate all four analytes from each other. When using reversed phase columns, such as C_8 or C_{18}, a high percentage ($\geq$80%) of aqueous mobile phase was required to retain HCTZ on the column due to the small molecule size and high polarity of HCTZ. Under these conditions, the sensitivity of HCTZ was poor and Losartan and

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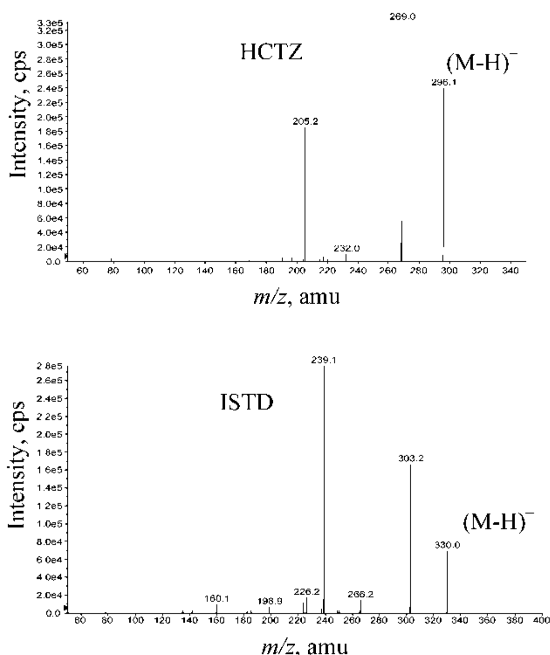

Figure 2. Product ion mass spectra of the de-protonated molecules of HCTZ and ISTD under the conditions used in the methods.

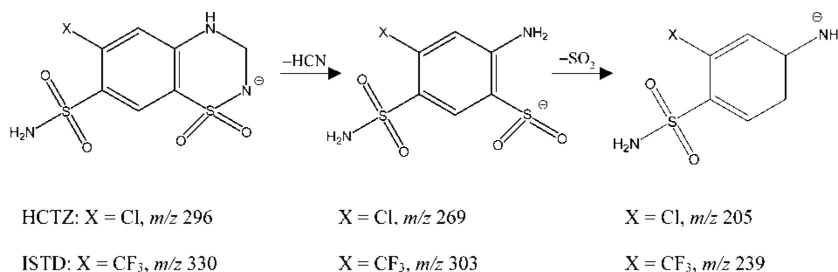

Figure 3. Proposed MS/MS fragmentation mechanisms of HCTZ and ISTD.

EXP-3174 were strongly retained on the columns. In addition, the MS/MS signal response of HCTZ was greatly reduced when Losartan and EXP-3174 co-eluted with HCTZ. Therefore, chromatographic separation of HCTZ from Losartan and EXP-3174 was critical to attain high MS/MS detection responses. After screening a number of columns from various vendors for the best separation results, a Restek Ultra Phenyl column (100×3.2 mm, 3 μm), protected by an Ultra Phenyl guard column (10×4 mm, 3 μm) and an in-line column filter (0.5 micron), was finally selected for the plasma and urine assay. An isocratic mobile phase consisting of ACN:10 mM ammonium acetate (40 : 60, v/v) was used to achieve a good compromise between peak shape and reasonable retention for HCTZ and ISTD (Fig. 4).

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Method Validation

Plasma Method

The plasma method was validated over the concentration range of 1 to 100 ng/mL. The lower limit of quantification (LLOQ) for HCTZ, defined as the lowest concentration on the standard line for which acceptable accuracy ($100 \pm 15\%$) and precision (coefficient of variation C.V. $<15\%$), was 1 ng/mL. Assessment of the intraday variability of the method was conducted in five different lots of human control plasma spiked with HCTZ over the calibration range of 1 to 100 ng/mL. The resulting precision and accuracy data are presented in Table 1. The intraday precision of the method, as measured by the coefficient of variation (% C.V.), was 4.1% at LLOQ, and was equal to or lower than 5.5% at all other concentrations used for the construction of the calibration curve. Accuracy was found to be within $\pm 7.9\%$ of the nominal concentration for all the standards evaluated. The correlation coefficient for the mean standard curves constructed from five different lots of human plasma was 0.995.

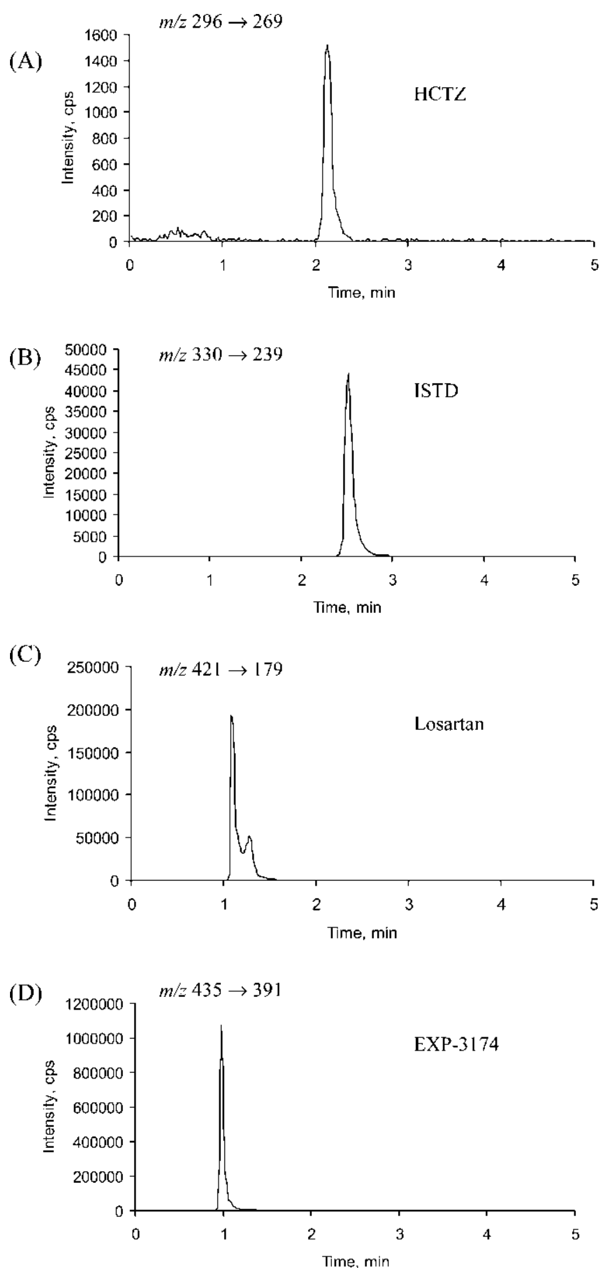

Figure 4. Representative HPLC-MS/MS chromatograms of a mixture of HCTZ (2 ng/mL), ISTD (100 ng/mL), Losartan (500 ng/mL), and EXP-3174 (1 μg/mL) standards, obtained by multiple reaction monitoring at m/z 296 → 269 for HCTZ (A); m/z 330 → 239 for ISTD (B); m/z 421 → 179 for Losartan (C); and m/z 435 → 391 for EXP-3174 (D).

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Table 1. Intraday precision and accuracy data for the determination of HCTZ in five different lots of human control plasma

Nominal concentration (ng/mL)	Mean[a] determined conc. (ng/mL)	Accuracy[b] (%)	Precision[c] (%)
1	1.03	103.0	4.1
2.5	2.37	94.8	4.1
5	4.79	95.8	5.0
10	9.78	97.8	2.5
25	24.65	98.6	5.5
50	51.11	102.2	2.5
100	107.9	107.9	1.4

[a]n = 5.
[b]Expressed as [(mean determined concentration)/(nominal concentration)] × 100.
[c]Coefficient of variation, n = 5.

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QC samples of HCTZ were prepared at three concentrations of 2 ng/mL (LQC), 40 ng/mL (MQC), and 80 ng/mL (HQC). The results of the intraday analysis (n = 5) of these QC samples are shown in Table 4. The precision (% C.V.) was ≤2.5% and accuracy was 94.1–95.8% at all three QC concentrations.

Table 2. Intraday precision and accuracy data for the determination of HCTZ in five different lots of human control urine

Nominal concentration (μg/mL)	Mean[a] determined conc. (μg/mL)	Accuracy[b] (%)	Precision[c] (%)
0.05	0.0507	101.4	3.8
0.2	0.192	96.0	0.5
0.5	0.481	96.2	1.6
1	0.98	98.0	2.1
2	1.97	98.5	1.5
5	5.07	101.4	1.1
10	10.23	102.3	1.6
20	21.21	106.1	0.9

[a]n = 5.
[b]Expressed as [(mean determined concentration)/(nominal concentration)] × 100.
[c]Coefficient of variation, n = 4.

Urine Method

The urine method was validated over the concentration range of 0.05 to 20 μg/mL. The LLOQ for HCTZ was 0.05 μg/mL. Assessment of the intraday variability of the method was conducted in five different lots of human control urine spiked with HCTZ over the calibration range of 0.05 to 20 μg/mL. The resulting precision and accuracy data are presented in Table 2. The intraday precision (% C.V.) of the method was 3.8% at LLOQ, and was equal to or lower than 2.1% at all other concentrations used for the construction of the calibration curve. Accuracy was found to be within ±6.1% of the nominal concentration for all the standards evaluated. The correlation coefficient for the mean standard curves constructed from five different lots of human urine was 0.998.

QC samples of HCTZ were prepared at three concentrations of 0.1 μg/mL (LQC), 9 μg/mL (MQC), and 18 μg/mL (HQC). The results of the intraday analysis (n = 5) of these QC samples are shown in Table 4. The precision (% C.V.) was ≤3.7% and accuracy was 93.9–100.7% at all three QC concentrations.

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Selectivity

Losartan and EXP-3174 were evaluated for the cross-talk in channels used for monitoring both HCTZ and the ISTD. Both Losartan and EXP-3174 were well separated from HCTZ and ISTD under the chromatographic condition utilized and no interference from these two compounds was observed in the MS/MS channels used for quantification of HCTZ and ISTD. Additionally, the cross-talk between channels used for monitoring both HCTZ and ISTD were evaluated by the analysis of plasma or urine samples containing HCTZ at the highest concentrations on the standard line and ISTD separately, and monitoring the responses in other MS/MS channels used for quantification. No response was observed in the channel of the other analytes at their retention times.

No peak eluting at the retention time of the analyte or ISTD was detected in samples from six different lots of human plasma or urine and pre-dose human plasma or urine samples, confirming method selectivity. Representative chromatograms of double-blank, human control plasma, or urine spiked with standards, and pre- and post-dose human plasma or urine samples are shown in Figures 5 to 8.

Extraction Recovery and Assessment of the Matrix Effect on Ionization

Extraction recovery and the effect of the plasma or urine matrix on ionization were evaluated for HCTZ using standards spiked at concentrations of 2.5, 10,

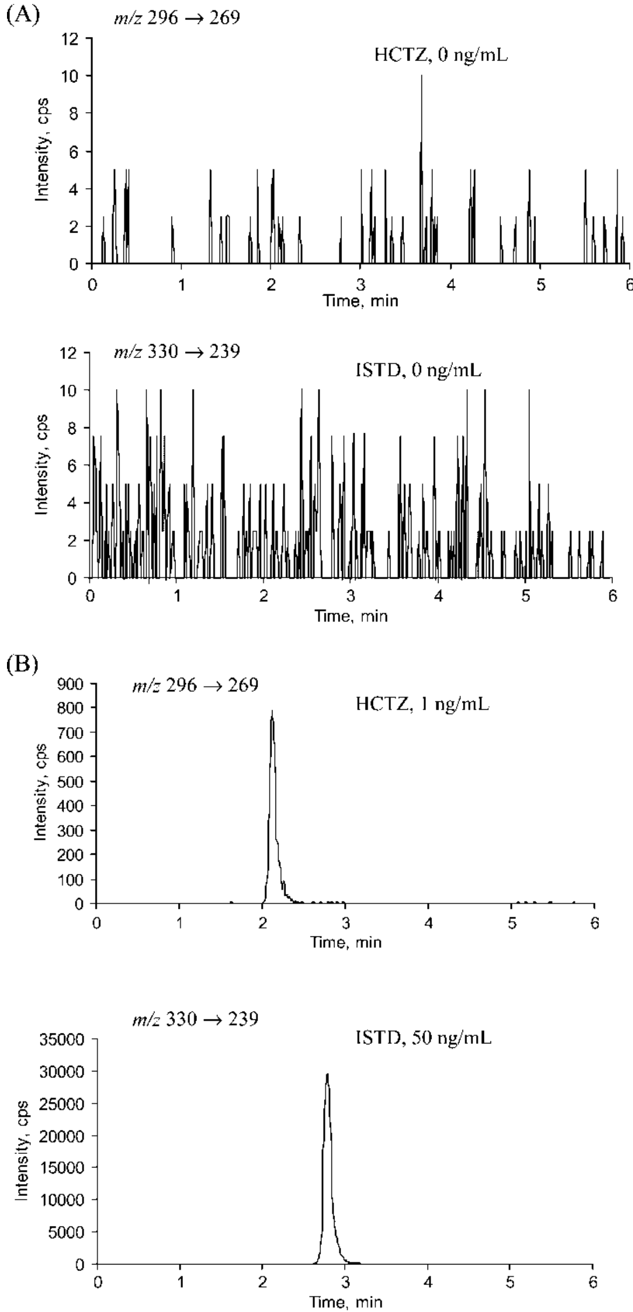

Figure 5. Representative HPLC-MS/MS chromatograms of: (A) human control plasma; (B) human control plasma spiked with 1 ng/mL of HCTZ and 50 ng/mL of ISTD.

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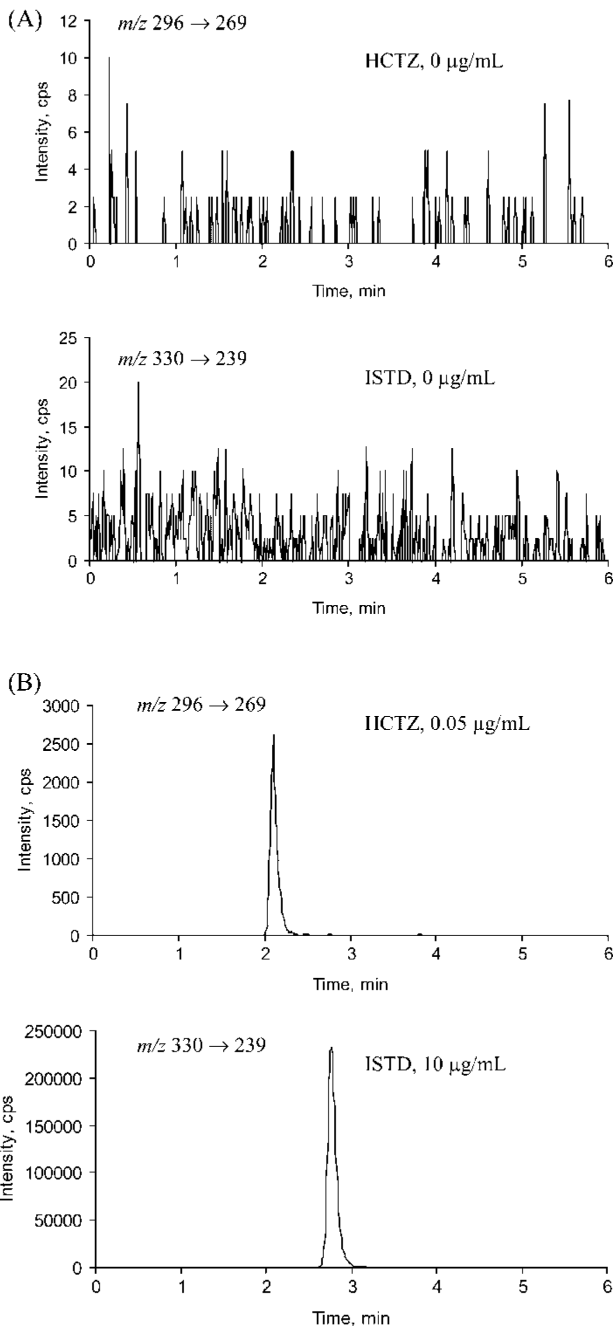

Figure 6. Representative HPLC-MS/MS chromatograms of: (A) human control urine; (B) human control urine spiked with 0.05 μg/mL of HCTZ and 10 μg/mL of ISTD.

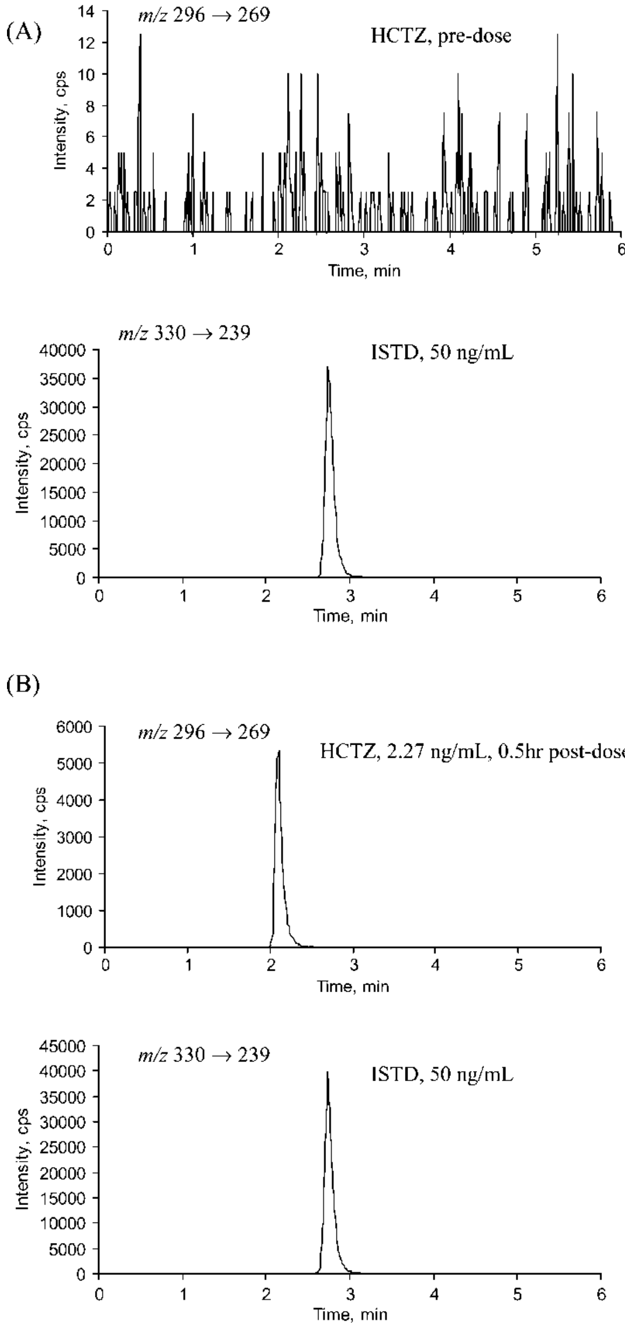

Figure 7. Representative HPLC-MS/MS chromatograms of: (A) pre-dose human plasma spiked with 50 ng/mL ISTD; (B) 0.5 hr post-dose human plasma (2.27 ng/mL) spiked with 50 ng/mL ISTD.

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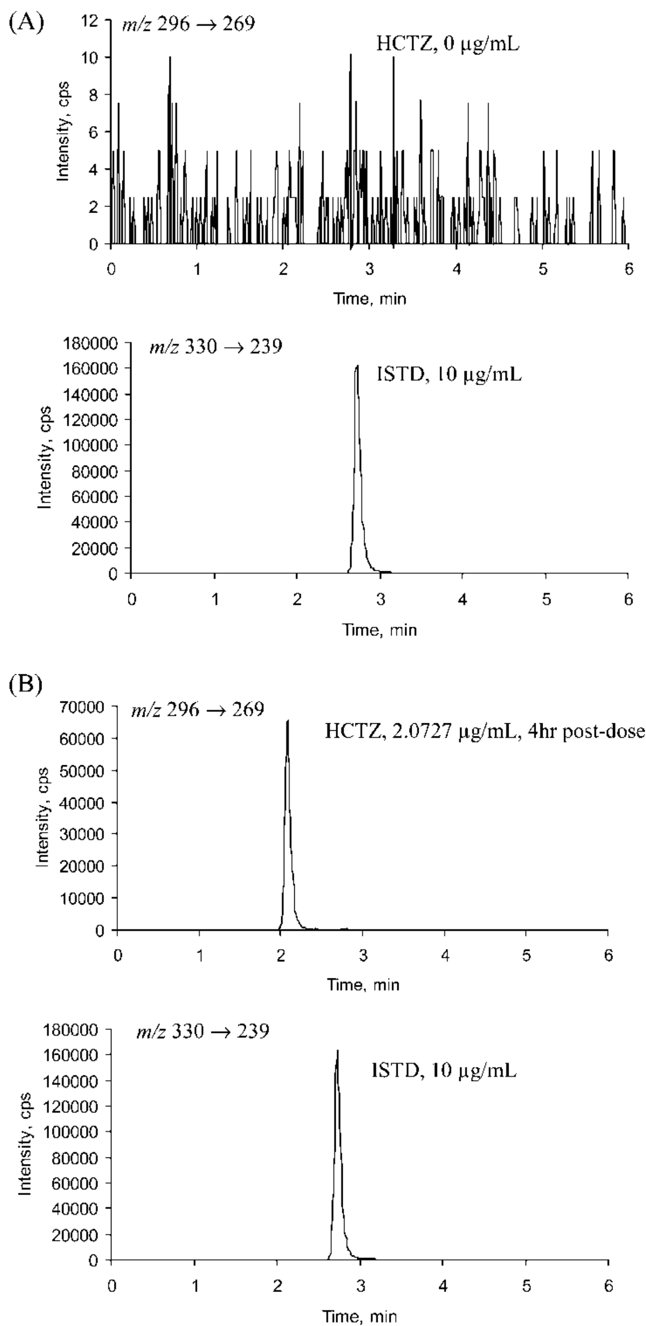

Figure 8. Representative HPLC-MS/MS chromatograms of: (A) pre-dose human urine spiked with 10 μg/mL of ISTD; (B) 4 hr post-dose human urine (2.0727 μg/mL) spiked with 10 μg/mL of ISTD.

and 50 ng/mL in plasma or 0.2, 2, and 10 μg/mL in urine, and for ISTD spiked at a concentration of 50 ng/mL in plasma or 10 μg/mL in urine. Recoveries of 96-well liquid-liquid extraction were determined by comparing the peak areas of standards spiked into five different lots of human control plasma and urine, and extracted as per sample preparation to human control plasma or urine extracted in the same manner, and then spiked post-extraction with a known amount of the drug. The mean recoveries of HCTZ and ISTD over the concentration range of the plasma method were >82.6 ($n = 15$) and 103.1% ($n = 15$), respectively. The mean recoveries of HCTZ and ISTD over the concentration range of the urine method were > 87.1 ($n = 15$) and 97.3% ($n = 15$), respectively.

The presence of any matrix effect may have an adverse impact on reliable analyte quantification, especially when a chemical analog rather than a stable isotope labeled compound is used as internal standard.[16,22] Verification of the absence of any significant "absolute" and "relative" matrix effects[16] between different human plasma or urine lots was confirmed experimentally. Matrix enhancement/suppression of ionization was evaluated by comparing the peak areas of human control plasma or urine samples extracted and then spiked with a known amount of each analyte, to neat standards injected directly in the same reconstitution solvent. For the plasma method, the average "absolute" matrix effect was +5.9% for HCTZ and +3.7% for ISTD. For the urine method, the average "absolute" matrix effect was +5.7% for HCTZ and +4.8% for ISTD. Based on the intraday precision and accuracy results (Tables 1 & 2) and the slope data (Table 3) that were obtained using five different lots of human control plasma or urine, the use of HFTZ as the internal

Table 3. Standard curve slope data for the determination of HCTZ in five different lots of human control plasma

	Slope	
Lot number	Human control plasma	Human control urine
1	0.0260	0.189
2	0.0249	0.189
3	0.0265	0.0186
4	0.0249	0.187
5	0.0250	0.190
Mean	0.0255	0.188
Standard dev.	0.000744	0.00164
Precision[a] (%)	2.9	0.9

[a]Coefficient of variation, $n = 5$.

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Table 4. Intraday analysis of QC samples containing HCTZ in human plasma and urine

	Low QC	Middle QC	High QC
Plasma			
Nominal conc. (ng/mL)	2	40	80
Mean determined conc. (ng/mL, n = 5)	1.91	38.31	75.30
Accuracy[a] (%)	95.5	95.8	94.1
Precision[b] (%)	2.5	0.8	1.9
Urine			
Nominal conc. (μg/mL)	0.1	9	18
Mean determined conc. (μg/mL, n = 5)	0.094	8.62	18.13
Accuracy[a] (%)	93.9	95.8	100.7
Precision[b] (%)	0.7	3.7	3.5

[a]Expressed as [(mean determined concentration)/(nominal concentration)] × 100.
[b]Coefficient of variation, n = 5.

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standard largely compensated for any variation in matrix effect and/or recoveries between the different lots of human control plasma or urine. Therefore, "relative" matrix effect on ionization from five different lots of human control plasma or urine was negligible.

Analyte Stability

Freeze-Thaw Stability

Human plasma and urine QC samples (n = 5 at each concentration) were subjected to three freeze-thaw cycles consisting of a thaw to reach room temperature and then refreezing at −20°C. These samples, together with a set (n = 5 at each concentration) of human QC samples that were not subjected to additional freeze-thaw cycles, were then defrosted and analyzed. In all cases, results for analyzing the QC samples of human plasma and urine that were subjected to these freeze-thaw cycles were within ±7.2, and ±8.2% of the nominal concentration, respectively.

Room Temperature Stability

Room temperature stabilities in human plasma or urine were confirmed for HCTZ by thawing three sets of QC samples and analyzing them immediately

after thawing, and then comparing the results with those generated from the analysis of the QC samples stored at room temperature for 5 hours before analyzing. Results indicated that HCTZ was stable in plasma or urine at room temperature for at least 5 hours.

Autosampler Stability

Autosampler stabilities in plasma or urine were ascertained by comparing the mean determined concentrations of QC samples (n = 3 at each concentration) analyzed at the beginning of a run with those of the same samples analyzed after 7 hours. In all cases, the results for analyzing the QC samples of human plasma and urine were within ±8.0 and ±7.2% of the nominal concentration, respectively.

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Reinjection and Dilution Reproducibility

Reinjection of the previously prepared samples may be considered in case that the original run failed due to an instrumental failure or unavailability of clinical samples for the repeat extraction and analysis. Reinjection reproducibility was confirmed by injecting and analyzing plasma or urine calibration standards twice within the time frame of 19 hours. For the plasma method, the precision (%C.V.) was 4.1 and 2.7% at LLOQ (1 ng/mL), and ≤5.5 and ≤4.1% at all other concentrations used for the construction of the calibration curve when the initially injected standard curve human plasma samples and reinjected standard curve samples were used for calculations, respectively. Assay accuracy was found to be within ±7.9 and ±8.6% of the nominal concentration for all the initially injected plasma standards and reinjected plasma standards, respectively. For the urine method, the precision (% C.V.) was 3.8 and 3.3% at LLOQ (50 ng/mL), and ≤2.1 and ≤2.5% at all other concentrations used for the construction of the calibration curve when the initially injected standard curve human urine samples and reinjected standard curve samples were used for calculations, respectively. Assay accuracy was found to be within ±6.1 and ±7.4% of the nominal concentration for all the initially injected urine standards and reinjected urine standards, respectively.

When the analyte concentrations in the post-dose samples were above the upper limit of the standard curve, samples were diluted with control plasma or urine prior to analysis. Sample dilution reproducibility was demonstrated by diluting and analyzing plasma samples prepared at a concentration of 1,000 ng/mL and urine samples at concentration of 160 μg/mL, respectively, and diluting each 11 times to within the standard curve range. After dilution, for plasma method, the accuracy of the diluted samples was 105.0% of the nominal standard concentration, and the precision (% C.V.) of three determi-

Table 5. Interday analysis of QC sample containing HCTZ in human plasma and urine

	Low QC	Middle QC	High QC
Plasma			
Nominal conc. (ng/mL)	2	40	80
Mean determined conc. (ng/mL, n = 16[a])	1.91	39.64	81.15
Accuracy[b] (%)	95.4	99.1	101.4
Precision[c] (%, n = 16)	4.6	4.0	5.3
Urine			
Nominal conc. (μg/mL)	0.1	9	18
Mean determined conc. (μg/mL, n = 12[d])	0.094	9.3	19.21
Accuracy[b] (%)	94.0	103.3	106.7
Precision[c] (%, n = 12)	4.3	4.3	3.6

[a]Over a period of 16 days.
[b]Expressed as [(mean determined concentration)/(nominal concentration)] × 100.
[c]Coefficient of variation.
[d]Over a period of 12 days.

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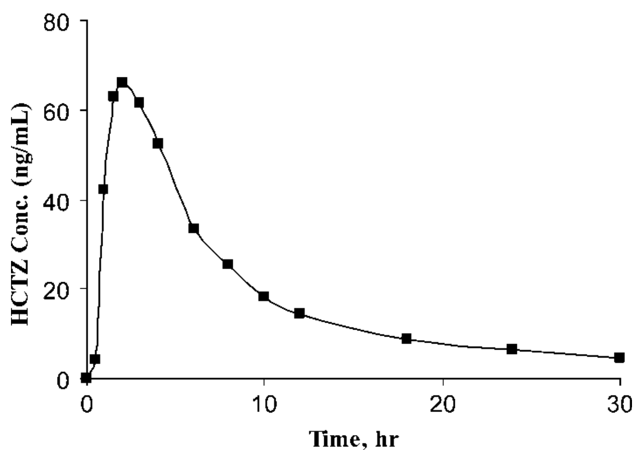

Figure 9. A representative plasma concentration (ng/mL) vs. time profile of HCTZ from subject #12 orally dosed with a COZAAR™ (100 mg Losartan) tablet and a HCTZ (12.5 mg) capsule.

Table 6. Results of urinary excretion of HCTZ from subject #12

Time (hr)	Total (mg)
Pre-dose	—
0–4	2.9610
4–8	0.1002
8–12	1.0250
12–18	0.1416
18–24	0.4263
24–36	0.4233
36–48	0.0247
Grand Total Amount (mg)	5.1021

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nations was 1.9%. For urine method, the accuracy of the diluted samples was 101.6% and the precision (% C.V., n = 3) was 3.3%.

Analysis of Clinical Samples

The plasma and the urine method have been applied successfully to the analysis of samples from a bioequivalence study. Interday precision and accuracy of both methods for the clinical studies were determined by analyzing QC samples at low, medium, and high concentrations. The QC samples, which were prepared before sample collection were analyzed in replicate with daily study samples. The results are showed in Table 5. The precisions (% C.V.) were $\leq$5.3 and $\leq$4.3%, and accuracies were 95.4–101.4% and 94.0–106.7% at all three QC concentrations of plasma and urine, respectively.

A representative plasma concentration - time profile of HCTZ from subject #12 orally dosed with a COZAAR^{TM} (100 mg Losartan) tablet and a HCTZ (12.5 mg) capsule is shown in Fig. 9. Total and individual urinary recoveries of HCTZ from the same subject are presented in Table 6.

CONCLUSIONS

HPLC-MS/MS methods were developed and validated for the determination HCTZ in human plasma and urine using a semi-automated liquid-liquid extraction method in a 96-well format. Only 400 and 100 μL of human plasma and urine were needed to achieve LLOQs of 1 and 50 ng/mL in human plasma and urine, respectively. Both methods were proven to be fast, sensitive, selective, precise, and accurate for the quantification of

HCTZ in the presence of Losartan and EXP-3174. The applicability of both methods was demonstrated by analyses of human plasma and urine clinical samples from human subjects participating in a bioequivalence study.

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