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Automated 96-well solid phase extraction and hydrophilic interaction liquid chromatography–tandem mass spectrometric method for the analysis of cetirizine (ZYRTEC®) in human plasma—with emphasis on method ruggedness

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Abstract

A high-throughput bioanalytical method based on automated sample transfer, automated solid phase extraction, and hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) analysis, has been developed for the determination of cetirizine, a selective H_1-receptor antagonist. Deuterated cetirizine (cetirizine-d_8) was synthesized as described and was used as the internal standard. Samples were transferred into 96-well plates using an automated sample handling system. Automated solid phase extraction was carried out using a 96-channel programmable liquid-handling workstation. Solid phase extraction 96-well plate on polymer sorbent (Strata X) was used to extract the analyte. The extracted samples were injected onto a Betasil silica column (50 × 3, 5 μm) using a mobile phase of acetonitrile–water–acetic acid–trifluroacetic acid (93:7:1:0.025, v/v/v/v) at a flow rate of 0.5 ml/min. The chromatographic run time is 2.0 min per injection, with retention time of cetirizine and cetirizine-d_8 both at 1.1 min. The system consisted of a Shimadzu HPLC system and a PE Sciex API 3000 or API 4000 tandem mass spectrometer with (+) ESI. The method has been validated over the concentration range of 1.00–1000 ng/ml cetirizine in human plasma, based on a 0.10-ml sample size. The inter-day precision and accuracy of the quality control (QC) samples demonstrated <3.0% relative standard deviation (R.S.D.) and <6.0% relative error (RE). Stability of cetirizine in stock solution, in plasma, and in reconstitution solution was established. The absolute extraction recovery was 85.8%, 84.5%, and 88.0% at 3, 40, and 800 ng/ml, respectively. The recovery for the internal standard was 84.1%. No adverse matrix effects were noticed for this assay. The automation of the sample preparation steps not only increased the analysis throughput, but also increased method ruggedness. The use of a stable isotope-labeled internal standard further improved the method ruggedness. Practical issues of analyzing incurred samples were discussed. This HILIC–MS/MS method for analysis of citirizine in human plasma was successfully used to support clinical studies.

Keywords: Cetirizine; Synthesis of cetirizine-d_8; HILIC–MS/MS; Method validation

1. Introduction

Cetirizine hydrochloride ((±)-[2-[4-[(4-chlorophenyl-methyl)]-1-piperazinyl]ethoxy]acetic acid, dihydrochloride), the active component of ZYRTEC® tablets and syrup, is an orally active and selective peripheral H_1-receptor antagonist

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[1]. Cetirizine is also an active metabolite of hydroxyzine, a first generation H_1-receptor antagonist [2]. In animal models, cetirizine shows negligible penetration into the brain and does not significantly occupy cerebral H_1 receptors. Thus, cetirizine has the advantage of lacking the CNS depressant effects frequently encountered in other antihistamines [3]. Cetirizine has a low degree of first-pass metabolism and is metabolized to a limited extend by oxidative *O*-dealkylation to a metabolite with negligible antihistaminic activity. The enzyme or enzymes responsible for this metabolism have remained unknown. Pharmacokinetic drug–drug interaction of cetirizine with pseudoephedrine, ketoconazole, erythromycin and azithromycin was not observed. However, a moderate 16% decrease in the clearance of cetirizine was observed when it was co-administered with theophylline. To meet the needs for pharmacokinetic studies, a rapid, selective, sensitive and robust analytical method is highly desirable. Analysis of cetirizine in biological fluids has been achieved using thin-layer chromatography [4], gas chromatography [5], HPLC with ultraviolet detection [6–12], and LC–MS/MS [13,14]. Cetirizine was also mentioned in a few articles specifically dealing with rapid multi-drug screening using LC–MS/MS [15–17] or GC–MS [18]. Methods using non-mass spectrometric detection may not have the desired selectivity and sensitivity. The methods designed for multi-drug screening usually compromise the individual analyte for the total screening capability and were usually not validated for quantitative bioanalysis. Eriksen et al. used a solid-phase extraction procedure on Oasis HLB sorbent and a reversed-phase gradient LC–MS/MS method with a run time of 6 min [14]. The low limit of quantitation is 5 ng/ml using 0.25 ml plasma sample. It should be mentioned that hydroxyzine was used as the internal standard. Hydroxyzine is also an antihistamine drug and is metabolized into cetirizine. Use of hydroxyzine as the internal standard might not be desirable for incurred sample analysis since the subjects may also take hydroxyzine. An isocratic reversed-phase LC–MS/MS method was described by de Jager et al. [13]. A simple protein precipitation using acetonitrile was used. The internal standard oxybutynin favorably coelutes with cetirizine and may compensate for matrix effects. And one should use caution since oxybutynin is a common drug used to relieve urinary and bladder difficulties.

In this article, a simple, fast and sensitive HILIC–MS/MS method is described for the analysis of as low as 1 ng/ml of cetirizine using 0.1-ml human plasma. The signal to noise ratio at 1 ng/ml is 35–1. The low limit of quantitation at 1 ng/ml is the lowest concentration required for a typical cetirizine pharmacokinetic study but lower detection could easily be achieved. The run time is only 2 min. A solid phase extraction (SPE) method that uses polymer sorbent was utilized to extract analyte and internal standard from plasma matrix. Chromatography was carried out on a silica column using an isocratic low aqueous/high organic mobile phase.

Particular effort has been employed in the method development to improve the method ruggedness during the incurred sample analysis and transferability. It has been well established in the industry that use of stable isotope-labeled internal standard can significantly improve the method ruggedness. A good internal standard should track the analyte during the extraction and compensate for any potential recovery inconsistency. It should elute close to the analyte on the column and compensate for potential inconsistent response due to matrix effects. Stable isotope internal standard (^{2}H, ^{13}C, or ^{15}N labeled analyte) is an ideal candidate for meeting the above criteria. Cetririzine-d_8 was therefore synthesized and used as the internal standard. Method ruggedness can also be improved by employing well-established automated sample preparation strategy and eliminating or minimizing labor-intensive and time-consuming manual operation [19–23]. Automated SPE using 96-well plate format has enjoyed the greatest success and is the leading industry trend for bioanalysis [19–27]. Here we report an HILIC–MS/MS method for analysis of cetirizine in human plasma that utilizes automated sample transfer and automated SPE based on 96-well plate format.

2. Experimental

2.1. Chemicals and reagents

Cetirizine (purity 98%) was purchased from ChemPacific Corporation (Baltimore, MA, USA), and internal standard cetirizine-d_8 (purity 95% with isotopic purity 100%) was synthesized in-house. The chemical structures of cetirizine and cetirizine-d_8 are shown in Fig. 1. Acetonitrile, methanol, dichloromethane, ethyl acetate, water, acetic acid, formic acid (all of LC grade), hydrochloric acid, sulfuric acid, sodium hydroxide, magnesium sulfate, sodium sulfate, potassium carbonate, sodium carbonate and silica were from Fisher Scientific (St. Louis, MO, USA). Trifluoroacetic acid (TFA) of LC grade, in 1 ml ampules, was from Sigma (St. Louis, MO, USA). Blank human plasma with sodium heparin as anticoagulant was from Biochemed (Winchester, VA, USA). 2-Chloroethyl-chloromethyl ether was obtained from TCI America (Portland, OR, USA). Copper(I)cyanide and 4-chlorobenzhydrylchloride were purchased from Acros Organics (Morris Plains, NJ, USA). Hydrogen chloride gas was from Aldrich Chemical (Milwaukee, WI, USA). Ethanol was from Aaper (Shelbyville, KY, USA). Piperazine-d_{10} was obtained from C/D/N Isotopes (Pointe-Claire, Quebec, Canada).

2.2. Synthesis and characterization of cetirizine-d_8

2.2.1. 2-Chloroethoxy acetonitrile (1)

2-Chloroethyl-chloromethyl ether (25 g, 194 mmol) was added to copper(I)cyanide (20 g, 223 mmol). The mixture was slowly heated to 65 °C while stirring. A sudden exotherm was noted. The flask was cooled with ice water to control the exotherm. After the exotherm subsided the mixture was heated to 100 °C for 2 h. A black suspension resulted. The

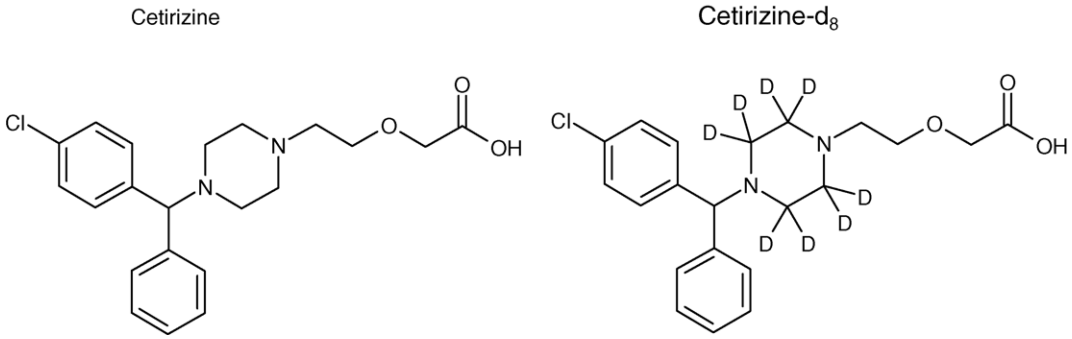

Fig. 1. Chemical structures of cetirizine and internal standard (IS) cetirizine-d_8.

product was obtained as a colorless oil (16.05 g) after Kugelrohr distillation at 120 °C at 0.01 mm Hg. The synthesis is outlined in Fig. 2.

2.2.2. 2-Chloroethoxy acetamide (2)

Concentrated sulfuric acid (6.3 ml, 114.8 mmol) was added drop wise over 20 min to 2-chloroethoxy acetonitrile (1) (8.366 g, 70 mmol) at 5–10 °C (ice bath cooling). An exotherm was noted. The clear colorless reaction mixture was stirred at 5–10 °C for 0.5 h and at room temperature overnight. The reaction mixture was poured into water (40 ml) at 0 °C. The mixture was stirred at 0–10 °C for 1 h. The mixture was extracted with dichloromethane (6 ml × 40 ml). The organic phase was dried over sodium sulfate, filtered and evaporated

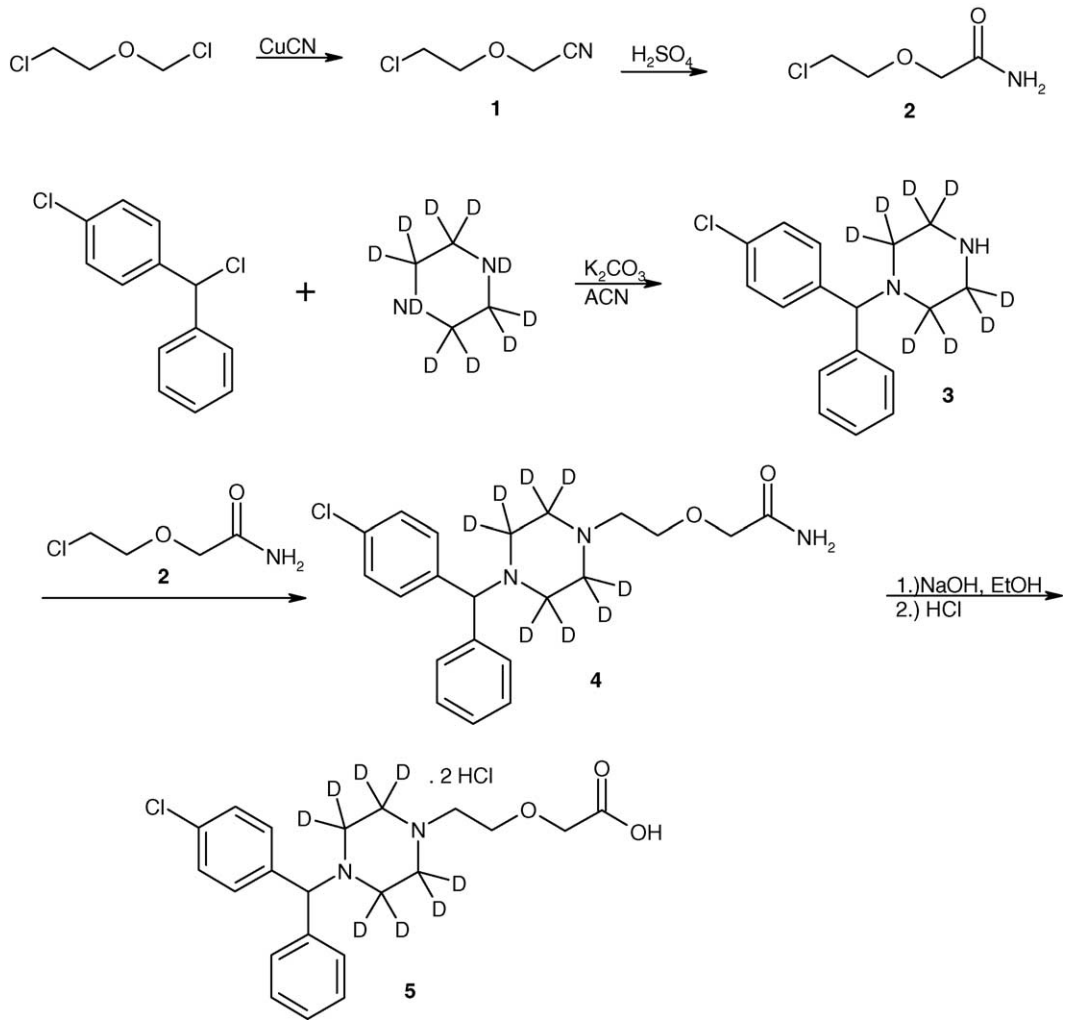

Fig. 2. Synthetic pathway of cetirizine-d_8.

to dryness at reduced pressure. The product was purified by crystallization from dichloromethane-ether (1/1, 50 ml). The product was obtained as a white solid (5.56 g, mp 72–75 °C) after drying on high vacuum.

2.2.3. 1-((4-Chlorophenyl)(phenyl)methyl)piperazine-d_8 (3)

To a solution of piperazine-d_{10} (1.923 g, 20 mmol) in acetonitrile (100 ml) was added potassium carbonate (5.528 g, 40 mmol). The resulting mixture was heated to reflux and a solution of 4-chlorobenzhydrylchloride (3.161 g, 13.33 mmol) in acetonitrile (50 ml) was added via addition funnel over 16 h. The reaction mixture was heated to reflux for a further 3 h. After cooling to room temperature insoluble materials were removed by filtration. The insoluble materials were washed with acetonitrile (50 ml). The combined organic solvents were evaporated to dryness at reduced pressure. The resulting oil was dissolved in ethylacetate (250 ml) extracted with water (1 ml × 50 ml), dried over sodium sulfate and filtered. A yellow oil (3.37 g) was obtained after the solvent was removed at reduced pressure.

2.2.4. 2-(2-(4-((4-Chlorophenyl)(phenyl)methyl) piperazin-d_8-1-yl)ethoxy)acetamide (4)

To a solution of 1-((4-chlorophenyl)(phenyl)methyl)piperazine-d_8 (3) (1.473 g, 5 mmol) in xylenes (10 ml) was added 2-chloroethoxy acetamide (2) (1.37 g, 10 mmol) and sodium carbonate (1.36 g, 12.8 mmol). The resulting suspension was heated to 110 °C (oil bath temperature) for 14 h. After cooling to room temperature the reaction mixture was diluted with xylene to 50 ml volume. Insoluble materials were removed by filtration. The solvent was removed at reduced pressure. The crude product was purified via flash chromatography on silica using methanol–dichloromethane–acetic acid (10/80/10) as mobile phase. The product was obtained as a brown semi solid (1.44 g).

2.2.5. Cetirizine-d_8 dihydrochloride (5)

To a solution of 2-(2-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-d_8-1-yl)ethoxy)acetamide (4) (1.44 g, 3.64-mmol) in ethanol (20 ml) was added sodium hydroxide powder (850 mg, 21.25 mmol). The resulting suspension was heated to reflux for 8 h. After cooling to room temperature the reaction mixture was diluted with water to 200 ml volume. The pH was adjusted to 4.5 with concentrated hydrochloric acid. The aqueous phase was extracted with chloroform (4 ml × 100 ml). The organic phase was dried over magnesium sulfate filtered and evaporated at reduced pressure to yield a brown oil. The oil was dissolved in toluene (20 ml) and hydrochloride gas was passed through the solution for 2 min. The solvent was removed at reduced pressure to yield an off white solid (510 mg). Electrospray ionization (ESI) MS m/z 397 $[M+H]^+$.

2.3. Calibration standards and quality control (QC) samples

Calibration standards and QCs were made from two separate stock solutions (0.5 mg/ml in methanol) of cetirizine. Prior to use, these two stock standard solutions were compared and approved within 5% of the HILIC–MS/MS response. The stock solutions were stored in polypropylene tubes with screw caps and were stable for at least 35 days when kept in a freezer at −70 °C and stable for at least 6 h when stored at room temperature. Pooled calibration standards at concentrations of 1.00, 2.00, 10.0, 20.0, 50.0, 200, 500, 900 and 1000 ng/ml were prepared in blank plasma pool made by combining 6 lots of blank plasma. QCs at levels of 3.00, 40.0, and 800 ng/ml were prepared for the determination of intraday and interday accuracy and precision. Over the curve QCs were prepared at 5000 ng/ml and low limit of quantitation (LLOQ) QCs were prepared at 1.00 ng/ml. The volumes of the spiking solutions were always kept below 2% of the plasma volumes. All standards and QCs were aliquoted into pre-labeled polypropylene vials and stored frozen at −20 °C and −70 °C. The long-term sample storage stability was determined by measuring the QCs that had been stored at −20 °C and −70 °C for 85 days against standards freshly prepared from stock solution. Prior to the sample analysis, the calibration standards were revised to 1.00, 2.00, 25.0, 50.0, 100, 125, 180, and 200 ng/ml and QCs were revised to 3.00, 75.0, and 150 ng/ml to better meet the analytical range. Over the curve QCs were prepared at 1000 ng/ml.

2.4. HILIC–MS/MS conditions

The HILIC–MS/MS system consisted of a Shimadzu 10ADVP HPLC system (Kyoto, Japan) and a PE Sciex API 3000 or API 4000 tandem mass spectrometer (Concord, Ontario, Canada) with (+) ESI. The analytical column, Betasil silica of 5 μm, 50 mm × 3.0 mm i.d., was from Keystone Scientific (Bellefonte, PA, USA). The columns were maintained at 30 °C. The mobile phase was acetonitrile–water–TFA–acetic acid (93:7:0.025:1, v/v/v/v). The flow rate was 0.5 ml/min. The back pressure was about 15 bar. The injection volume was 3 μl and run time was 2.0 min. The sample tray temperature was kept at 8 °C. The injection wash solution was acetonitrile containing 0.1% formic acid. The needle was rinsed prior to and after each injection with 0.50 ml of the wash solution. Autosampler carry-over was determined by injecting the highest calibration standard then an extracted blank sample. No carry-over was observed, as indicated by the lack of either cetirizine or internal standard peak in the blank sample. The background noise in the blank sample was also not elevated. The silica column demonstrated excellent stability. One column was typically used for at least 500 injections of extracted samples.

Sensitivity of the multiple reaction monitoring (MRM) was optimized by testing with an infusion of 0.1 μg/ml ceti-

rizine in a mixture of acetonitrile and water (1:1, v/v). On the API 3000, the Ionspray needle was maintained at 2.5 kV. The turbo gas temperature was 350 °C and the auxiliary gas flow was 8.0 L/min. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 8, 6, and 4, respectively. The declustering potential (DP) and focusing potential (FP) were at 35 V and 190 V, respectively. The mass spectrometer was operated under MRM mode with a collision energy (CE) of 12 eV. The transitions (precursor to product) monitored were *m/z* 389 → 201 for cetirizine, and 397 → 201 for IS. The dwell time was 200 ms for cetirizine and 100 ms for internal standard. Both quadrupoles were maintained at unit resolution. Chromatograms were integrated using the Analyst version 1.1 software. A weighted 1/concentration^2 linear regression was used to generate calibration curves from standards and calculate the concentrations of quality control samples.

2.5. Sample preparation

Samples were thawed unassisted, vortex-mixed for 20 s on a multi-tube vortexer, and centrifuged at 1130 × *g* at room temperature for 10 min on a Beckman Coulter J2-HS centrifuge (Beckman Coulter, Fullerton, CA, USA). The samples were arranged according to the final injection sequence. 0.1 ml was then transferred from vials into 1-ml 96-well deep-well collection plate (Axygen, Union City, CA, USA) using a MultiProbe™ II automated sample handling system (Packard Instrument Company, Meriden, CT, USA) controlled by WinPrep software. An aliquot of 50 μl of IS solution (cetirizine-d_8, 80 ng/ml in 1:1 acetonitrile–water) was then added to all samples (except blanks). An aliquot of 150 μl of 1% TFA in water was added to all samples. The sample plate was then manually transferred to a Tomtec Quadra™ 96-320 robot (Tomtec, Hamden, CT, USA) and automated SPE was carried out. The sample solution in the 96-well collection plate was mixed by aspirating and dispensing for 10 times using the Tomtec. Then 0.30 ml of the sample solution was transferred onto the SPE plate (Strata × 33 μm polymeric sorbent, 10 mg/well) from Phenomenex (Torrance, CA, USA), which had been conditioned with 0.50 ml of methanol followed by 0.50 ml of water, both by gravity. The sample solutions were allowed to elute by gravity for 10 min and then by very low pressure (approximately 0.5 scfh) by Speedisk® 96 pressure processor from J.T. Baker (Phillipsburg, NJ). The SPE sorbents were then washed with 2% formic acid in water, followed by 5% methanol in water. For both wash steps, low pressure was applied to let the solvents pass. The SPE plate was then placed on top of a clean Axygen 96-well deep-well collection plate. 200 μl of acetonitrile was added to each SPE well and solvent was allowed to elute by gravity for 5 min. Another 200 μl of acetonitrile was added to each SPE well and minimal pressure was applied to elute the solvent. The eluent was evaporated to dryness by a stream of nitrogen in a Speed Dry evaporator at 50 °C, which took about 15 min. The residue was reconstituted with 400 μl of acetonitrile. The collection plate was sealed with sealing mats (dimpled) for 96 deep-well plate from Axygen.

2.6. Validation of the HILIC–MS/MS method

The method was validated by three consecutive analytical runs on three separate days. Each run contained a single set of calibration standards and six replicates of QCs at each concentration level. One run also included lower limit of quantitation QCs (1.00 ng/ml), and over the curve QCs (5000 ng/ml), which were diluted 10-fold with control blank plasma prior to analysis. Each run also contained other test samples such as processing and storage stability samples. Calibration standards, QCs and other test samples were randomized through the run. An extracted blank sample was always placed after the upper limit of quantitation to determine carry-over of the HILIC–MS/MS system. One run contained 96 samples to simulate the length of clinical sample analysis. The method specificity was evaluated by screening six lots of blank plasma. These lots were spiked with cetirizine at 0.000, and 40.0 ng/ml. The spiked samples were extracted and analyzed to confirm lack of interference and absence of lot-to-lot variation. Analyte stability was tested by subjecting QCs through storage at −20 °C and −70 °C, multiple freeze thaw cycles, and on the bench at room temperature. Post-extraction analyte stability was also determined. Extraction recovery was determined by comparing the peak areas of the analyte and internal standard extracted from plasma with those of post-extraction spiked samples at the corresponding concentrations.

The method ruggedness was tested by additional validation run carried out by the chemists performing incurred sample analysis. For the incurred samples, the calibration curve was truncated to 1–200 ng/ml since most of the incurred samples are below 200 ng/ml and additional validation run was performed. A representative chromatogram of incurred samples shows in Fig. 3.

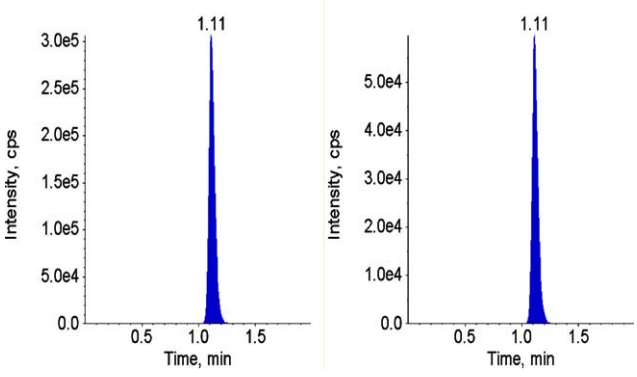

Fig. 3. Chromatogram of a representative incurred plasma sample spiked with cetirizine-d_8. Column: Betasil silica 50 mm × 3 mm, i.d., 5 μm; mobile phase: acetonitrile–water–TFA–acetic acid (93:7:0.025:1, v/v/v/v); flow rate: 0.5 ml/min; injection volume: 3 μl.

The specificity of this method against pseudoephedrine, a commonly coadministered drug, was determined by spiking the QCs with pseudoephedrine at 300 ng/ml. Those QCs were measured against calibration standards and results were compared to the normal QCs.

3. Results and discussion

3.1. HILIC–MS/MS

Positive ion ESI mass spectrum of ion scan for cetirizine shows $[M+H]^+$ at 389 and a dominant product ion at 201. This is the same as reported by the literatures [13,14]. Positive ion ESI mass spectrum of ion scan for cetirizine-d_8 is shown in Fig. 4. The $[M+H]^+$ at 397 was the predominant ion in the Q1 spectrum of cetirizine-d_8, which was used as the precursor ion for obtaining MS/MS product-ion spectrum. The dominant product ion is also at 201. The fragmentation is shown in Fig. 4.

The selection of a silica column and aqueous-organic mobile phase for quantitative analysis of cetirizine was based on our previous experiences for analyzing other polar compounds in biological fluids [28–32]. Bare silica columns operated with low aqueous–high organic mobile phases are a viable means of analyzing polar compounds in biological fluid. Mobile phases containing highly organic solutions would lead to favorable spraying conditions at the LC–MS interface necessary for adequate sensitivity [33]. Even though TFA was reported to suppress electrospray signals due to its ion-pairing in the gas phase with the analyte ions [34], the gain of sensitivity by using higher organic content was so large that the mobile phase containing small amounts of TFA (0.025%) still yield good signal to noise ratio (and peak shape). This has been observed for other analytes when HILIC–MS/MS methods were used [30]. Moreover, in our laboratory, it was found that the ionization suppression due to TFA could be avoided through the addition of 1% acetic acid in the mobile phase [35]. The greater overall sensitivity was achieved with the mobile phase containing high organic content optimized with the addition of acetic acid and TFA. Fig. 5 shows the chromatograms of three consecutive injections of cetirizine samples (extracted calibration standard at 1000 ng/ml, extracted blank, and extracted low limit of quantitation at 1 ng/ml) on a silica column. Minimal carryover from autosampler was observed occasionally. It accounts less than 5% of lower limit of quantitation. Good signal to noise ratio (>35) was observed for the cetirizine LLOQ peak. Fig. 6 shows the chromatogram of blank plasma spiked with only cetirizine-d_8. No interference from cetirizine-d_8 to the analyte was observed. Cetirizine and cetirizine-d_8 were well retained on column; its capacity factor (k') is 3.0 on the silica column. On the silica column, when the TFA concentration was kept constant at 0.025%, increasing acetonitrile concentration in the mobile phases not only increased cetirizine on column retention, as predicted from the HILIC mechanism, but also enhanced its signal intensity due to the more favorable spraying condition with a mobile phase of higher organic content. Recently, scientists from other institutes have also demonstrated that HILIC–MS/MS bioanalytical meth-

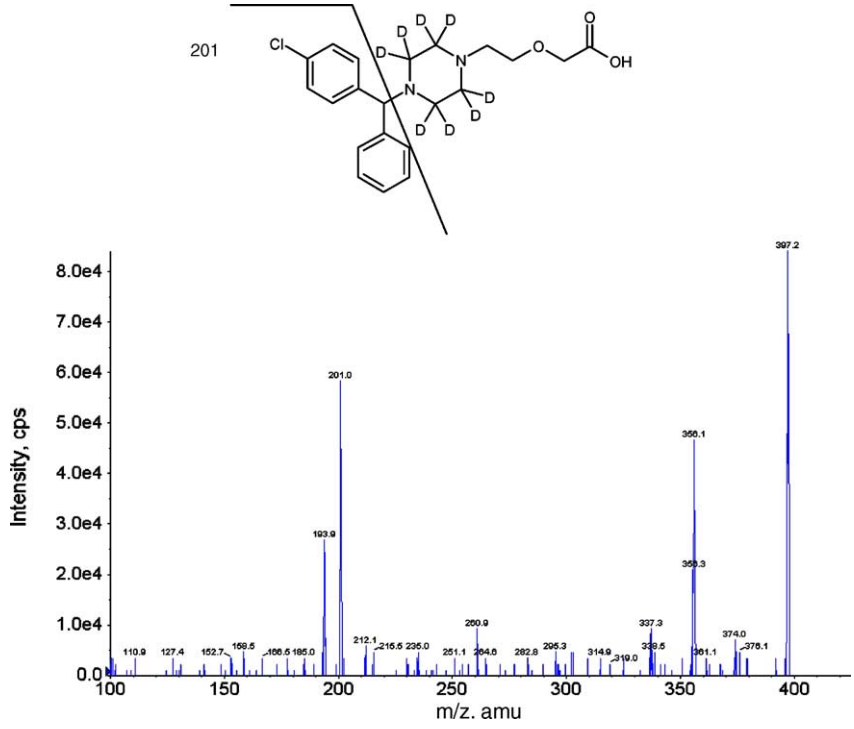

Fig. 4. Initial product ion scan mass spectra of the protonated molecule of cetirizine-d_8.

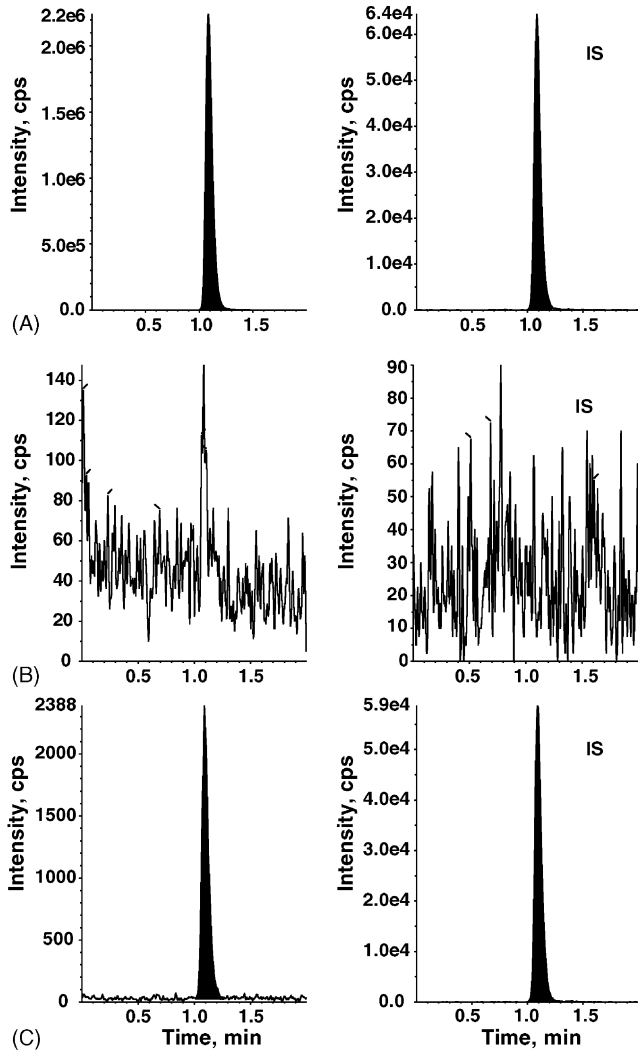

Fig. 5. Chromatogram of three consecutive injections of (A) extracted calibration standard at 1000 ng/ml of cetirizine, (B) extracted blank plasma sample, and (C) extracted low limit of quantitation at 1.00 ng/ml of cetirizine. IS: Internal standard (cetirizine-d_8); column: Betasil silica 50 mm × 3 mm, i.d., 5 μm; mobile phase: acetonitrile–water–TFA–acetic acid (93:7:0.025:1, v/v/v/v); flow rate: 0.5 ml/min; injection volume: 3 μl.

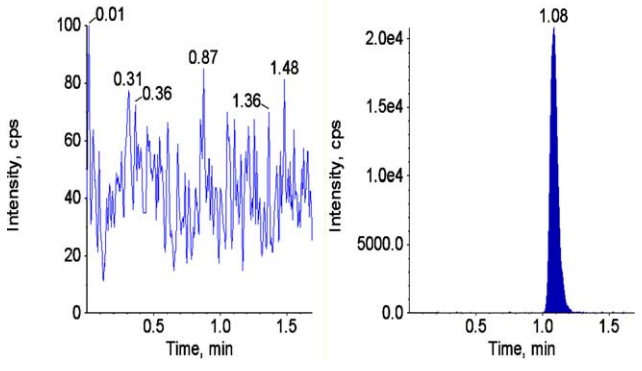

Fig. 6. Chromatogram of extracted blank plasma spiked with cetirizine-d_8 only. Column: Betasil silica 50 mm × 3 mm, i.d., 5 μm; mobile phase: acetonitrile–water–TFA–acetic acid (93:7:0.025:1, v/v/v/v); flow rate: 0.5 ml/min; injection volume: 3 μl.

ods would be ideal for analyzing polar compounds in biological fluids [36–39].

3.2. Extraction

In the current method, the Multiprobe™ II was programmed to aliquot samples from individual vials to a 96-well plate and to add internal standard. Sample transfer is one of the major time-consuming steps during the sample extraction process. The use of a single robotic liquid handler with either four or eight tips (i.e. Packard Multiprobe™ II) was ideal for the sample transfer. Before use, each of the four tips on the Multiprobe™ II was checked for accuracy and precision at 50 μl and 450 μl by pipetting and weighing water. The relative standard deviation (R.S.D.) and relative error (RE) values were below 5% for each tip. Furthermore, the good precision for pipetting plasma samples was reflected by the very low R.S.D. values for the calibration standards and QCs.

A Tomtec Quadra™ 96 workstation equipped with 96 tips is capable of extracting 96 samples simultaneously and would be ideal for sample extraction. Solid phase extraction based on reversed-phase mechanism using sorbents such as C18 or polymers is ideal for HILIC–MS/MS since two orthogonal analyte extraction mechanisms are involved in the sample clean-up. Since cetirizine possess both amine and carboxylic acid groups, TFA is added to the sample prior to the extraction. Addition of TFA keeps the carboxylic acid group unionized and also forms ion-pair with the amine group, both increasing the hydrophobicity of the analyte and increasing extraction recovery on the polymer SPE sorbent. Consistent extraction recoveries were obtained for all three levels of concentration investigated. The recovery is 85.8% (R.S.D.% = 10.7, n = 6) at 3 ng/ml, 84.5% at 40 ng/ml (R.S.D.% = 2.6, n = 6), and 88.0% (R.S.D.% = 4.6, n = 6) at 800 ng/ml, respectively. The recovery for the internal standard was 84.1% (R.S.D.% = 4.2, n = 18). Similar strategy has been successfully used to extract morphine glucuronide metabolites from plasma on reversed-phase C18 SPE [40].

3.3. Validation results

Six lots of blank control plasma were tested for matrix interference. The regions of the analyte and internal standard peaks were free from interference. Matrix effects from co-eluting endogenous components in biological fluids have been well documented in the literature for compromising the reproducibility and accuracy of the analysis [41]. When the samples were spiked with cetirizine at 40 ng/ml, the relative standard deviation (R.S.D.) and relative error (RE) were 5.1% and +0.6%, respectively as shown in Table 1. These tight R.S.D. and RE values indicate no significant lot-to-lot variation in matrix effects. Consistent peak areas for both cetirizine and internal standard were obtained for all six lots of plasma. Suppression caused from cetirizine to internal standard and

Table 1
Matrix lot-to-lot reproducibility

	Theoretical concentration (ng/ml)		HILIC–MS/MS peak area			
	0.000	40.0	0.000		40.0	
	Measured concentration (ng/ml)		Cetirizine	IS	Cetirizine	IS
Matrix lot #						
1	0.00	42.5	0	86923	126072	81275
2	0.00	39.6	0	84271	124663	86393
3	0.00	40.0	0	83963	122881	84132
4	0.00	36.7	0	87145	107351	80202
5	0.00	41.7	0	85355	119431	78549
6	0.00	41.0	0	91389	115744	77455
Mean	0.00	40.3	0	86508	119357	81334
R.S.D. (%)	N/A	5.1	N/A	3.2	5.8	4.2
RE (%)	N/A	+0.6	N/A	N/A	N/A	N/A

IS: Internal standard (cetirizine-d_8); R.S.D.: relative standard deviation; RE: relative error calculated as [(measured value/nominal value) × 100%] − 100%; N/A: not applicable.

vice verse was not observed. A good internal standard should track the analyte during the extraction and compensate for any potential recovery inconsistency. It should elute close to the analyte on the column and compensate for potential inconsistent response due to matrix effects. Internal standard should not cause interference or ion suppression to the analyte and vice versa. Stable isotope internal standard (^{2}H, ^{13}C, or ^{15}N labeled analyte) is an ideal candidate for meeting the above criteria. Cetirizine-d_8 as the internal standard works well for this assay.

Calibration curve parameters and data are listed in Table 2. The correlation coefficients of the three validation curves were all >0.998. The slopes are consistent throughout the validation batches. The standards show a linear range of 1–1000 ng/ml, using weighted (1/concentration^2) least-square linear regression. The precision and accuracy data for QC samples are summarized in Table 3. The data show that this method is consistent and reliable with low R.S.D.s and REs values. For the LLOQ QCs, the R.S.D. ($n = 6$) of the measured concentration was 7.0%. The relative error of the mean of the measured concentrations were +4.0%.

The stability tests were designed to cover the anticipated conditions that the clinical samples may experience. Stabilities of sample processing (freeze/thaw, bench-top and storage), and chromatography (extracts) were tested and established. The results are summarized in Table 4. Five freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to analysis, appeared to have little effect on the quantitation. QC samples stored in freezers at −20 °C and −70 °C remained stable for at least 85 days. Extracted calibration standards and QC samples were allowed to stand at 2–8 °C for 47 h prior to injection. No effect on quantitation of the calibration standards or QC samples was observed.

Table 2
Precision and accuracy of calibration standards ($n = 3$)

	Concentration (ng/ml)									Slope	r^2
	1.00	2.00	10.0	20.0	50.0	200	500	900	1000		
Batch 1	0.945	2.22	9.85	20.5	47.9	197	475	932	1010	0.0363	0.9984
Batch 2	0.950	2.22	9.50	20.5	46.4	205	481	939	1010	0.0368	0.9980
Batch 3	0.974	2.11	9.86	20.6	45.9	193	486	960	1030	0.0358	0.9986
Mean	0.956	2.18	9.74	20.5	46.7	198	481	944	1020	0.0363	0.9983
R.S.D. (%)	1.6	2.9	2.1	0.3	2.2	3.1	1.1	1.5	1.1	1.4	
RE (%)	−4.4	+9.0	−2.6	+2.5	−6.6	−1.0	−3.8	+4.9	+2.0		

Table 3
Precision and accuracy of quality control samples

	Concentration (ng/ml)							
	Intraday ($n = 6$)					Interday ($n = 18$)		
	1.00	3.00	40.0	800	5000[a]	3.00	40.0	800
Mean	1.04	2.85	38.2	773	4620	2.83	37.6	760
R.S.D. (%)	7.0	1.5	1.3	0.8	2.3	2.1	3.0	1.8
RE (%)	+4.0	−5.0	−4.5	−3.4	−7.6	−5.7	−6.0	−5.0

[a] Samples were diluted ten fold with blank plasma prior to analysis.

Table 4
Stability of the samples ($n = 6$)

	Concentration (ng/ml)		
	3.00	40.0	800
5 freeze/thaw cycles			
Mean	2.89	38.4	764
R.S.D. (%)	1.7	1.6	1.7
RE (%)	−3.7	−4.0	−4.5
24 h bench-top			
Mean	2.72	36.8	753
R.S.D. (%)	3.6	1.0	2.0
RE (%)	−9.3	−8.0	−5.9
−20 °C for 85 days			
Mean	3.35	41.8	812
R.S.D. (%)	3.6	1.9	1.3
RE (%)	+11	+4.5	+1.5
−70 °C for 85 days			
Mean	3.13	39.9	791
R.S.D. (%)	2.8	1.8	2.3
RE (%)	+4.3	−0.3	−1.1
47 h extract			
Mean	2.71	38.2	758
R.S.D. (%)	1.8	2.2	2.5
RE (%)	−9.7	−4.5	−5.3

Since most of the samples had concentrations below 200 ng/ml, it was decided to perform an additional validation to truncate the curve range. The validated QC concentrations should mimic those found in incurred samples [42]. The method was therefore revised to truncate the curve range to 1.00–200 ng/ml. An additional validation batch was performed and the QC results are shown in Table 5. Since pseudoephedrine is often coadministered with cetirizine, method specificity was established by analyzing the QCs fortified with pseudoephedrine. The results shown in Table 6 clearly indicate this method is specific and is not biased by the presence of pseudoephedrine. The peak area responses of both cetirizine and cetirizine-d_8 from the specificity QCs are almost identical to those in regular QCs. The method robustness was demonstrated by using multiple analytical columns and HILIC–MS/MS instruments over the course of the sample analysis. The QC performance for the entire sample analysis

Table 5
Precision and accuracy of quality control samples for the revised curve range

	Concentration (ng/ml)				
	Intraday ($n = 6$)				
	1.00	3.00	75.0	150	1000[a]
	1.13	3.11	74.1	150	1010
	1.05	3.11	78.4	153	1030
	1.09	3.07	76.8	153	1010
	1.04	3.10	75.1	151	1010
	1.06	3.15	75.5	153	1020
	1.05	3.23	75.8	154	1000
Mean	1.07	3.13	76.0	152	1013
R.S.D. (%)	3.2	1.6	1.8	0.9	0.9
RE (%)	+7.0	+4.3	+1.3	+1.3	+1.3

[a] Samples were diluted ten fold with blank plasma prior to analysis.

Table 6
Precision and accuracy of quality control samples for the specificity test (QCs were spiked with pseudoephedrine at 300 ng/ml)

	Concentration (ng/ml)		
	3.00	75.0	150
	3.07	77.0	151
	3.18	76.9	153
	3.23	77.2	155
Mean	3.16	77.0	153
R.S.D. (%)	2.6	0.2	1.3
RE (%)	+5.3	+2.7	+2.0

is monitored and all of the QCs in all batches ($n = 14$) passed the acceptance criterion (85–115% of nominal values). The internal standard responses are consistent throughout the run for QCs and incurred samples.

4. Conclusion

A sensitive, reliable and rugged HILIC–MS/MS method for the measurement of cetirizine in human sodium heparin plasma has been successfully developed and validated. Automated sample aliquoting and SPE extraction procedures on the 96-well format were employed. Cetirizine-d_8 was used as the internal standard. The LLOQ is 1.00 ng/ml for cetirizine using only 0.10 ml plasma. The run time is only 2 min. Practical considerations of method validation were discussed. This validated method has been successfully used to measure cetirizine in human plasma from a drug–drug interaction study.

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