



Original Research Article

Fast and sensitive LC–MS/MS method for the simultaneous determination of lisinopril and hydrochlorothiazide in human plasma

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ABSTRACT

A sensitive and rapid liquid chromatography-tandem mass spectrometry (LC–MS/MS) method has been developed for the simultaneous determination of lisinopril (LIS) and hydrochlorothiazide (HCTZ) in human plasma using their labeled internal standards (ISs). Sample pre-treatment involved solid phase extraction on Waters Oasis HLB cartridges using 100 μ L of plasma, followed by liquid chromatography on Hypersil Gold C₁₈ (50 mm \times 3.0 mm, 5 μ m) column. The analytes were eluted within 2.0 min using acetonitrile-5.0 mM ammonium formate, pH 4.5 (85:15, v/v) as the mobile phase. The analytes and ISs were analyzed in the negative ionization mode and quantified using multiple reaction monitoring. The method showed excellent linearity over the concentration range of 0.50–250.0 ng/mL for both the analytes. The intra-batch and inter-batch precision (% CV) was \leq 5.26% and their extraction recoveries were in the range of 96.6%–103.1%. Matrix effect evaluated in terms of IS-normalized matrix factors ranged from 0.97 to 1.03 for both the analytes. The validated method was successfully applied to determine the plasma concentration of the drugs using 10 mg lisinopril and 12.5 mg hydrochlorothiazide fixed dose formulation in 18 healthy Indian volunteers.

1. Introduction

Hypertension is one of the major risk factors for stroke and various cardiovascular diseases. The current measures for blood pressure (BP) control in patients with hypertension are far from optimal, mainly due to poor adherence to prescribed antihypertensive drug therapies and associated side effects [1]. It is now increasingly evident that monotherapy is unable to adequately control BP in a majority of the patients. Thus, fixed dose combination (FDC) therapy with two different classes of antihypertensive agents can significantly help in lowering their dose strength and thereby reducing their side effect profiles [1,2]. In combination therapy, one component is generally a diuretic drug together with an angiotensin converting enzyme (ACE) inhibitor, angiotensin receptor blocker or beta-blocker. In this regard, combination of an ACE inhibitor, like lisinopril (LIS), with a diuretic agent hydrochlorothiazide (HCTZ) is recognized as an effective option as these two drugs exhibit complimentary mechanism of action and as a result give an additive antihypertensive effect. Drugs belonging to the class of ACE inhibitors suppress the endogenous conversion of angiotensin I to angiotensin II and thus control elevation in blood pressure [3,4].

Several methods are available to determine LIS as a single analyte in human plasma or urine by high-performance liquid chromatography (HPLC) with fluorescence [5,6], ultraviolet (UV) [6] or mass detection [7–13]. Few other methods present simultaneous determination of LIS together with other histamine H₂ antagonists [14] and other anti-hypertensive medications [15,16] in pharmaceutical formulations and human serum. Similarly, numerous methods are reported for the estimation of HCTZ in human plasma alone [17–21] or in combination with other antihypertensive drugs like bisoprolol [22], candesartan [23], irbesartan [24,25], amlodipine and valsartan [26], aliskiren and amlodipine [27], losartan and losartan acid [28], ramipril [29] and telmisartan [30]. So far only few reports are available for the simultaneous determination of LIS and HCTZ in biological samples [31,32]. Elsebaei and Zhu [31] developed a fast gradient HPLC-UV method for the simultaneous determination of seven ACE inhibitors including LIS together with HCTZ in spiked human plasma and urine. Yang and co-workers [32] studied the pharmacokinetics of LIS and HCTZ after administration of single and multiple doses of their combination formulation in healthy Chinese subjects. They employed two different methods based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis for LIS and HPLC-UV for HCTZ.

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Moreover, information concerning method development and validation presented was very limited.

To the best of our knowledge there are no reports on the pharmacokinetics of this potent drug combination in Indian subjects. Thus the objective of the present work was to develop and validate a sensitive, selective and rapid LC–MS/MS method in the negative ionization mode for simultaneous analysis of LIS and HCTZ in human plasma. The method was successfully applied to a bioequivalence study of 10/12.5 mg LIS/HCTZ FDC formulation in healthy Indian subjects.

2. Experimental

2.1. Chemicals and materials

Reference standards of LIS (99.15%), HCTZ (99.88%) and labeled internal standards (ISs) lisinopril-d5 (99.53%) and hydrochlorothiazide-13C,d2 (99.29%) were obtained from Clearsynth Labs Pvt. Ltd. (Mumbai, India). HPLC-grade acetonitrile was procured from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). Ammonium formate and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oasis HLB (1 mL, 30 mg) extraction cartridges were from Waters Corporation (Milford, MA, USA). Water used in the study was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma in K₃EDTA was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20 °C until use.

2.2. Liquid chromatography and mass spectrometry conditions

A Shimadzu HPLC system (Kyoto, Japan) consisting of binary LC-20 CE prominence pump, autosampler (SIL-HTc), a solvent degasser (DGU-20A₃ Prominence) and temperature-controlled compartment for column (CTO-10AS_{VP}) was used for reversed-phase chromatographic analysis. The chromatographic separation of the analytes was carried out at 30 °C using Hypersil Gold C₁₈ (50 mm×3.0 mm, 5 μm) column from Thermo Scientific (Cheshire, UK). A mixture of acetonitrile and 5.0 mM ammonium formate (pH 4.5 adjusted with 0.1% formic acid) (85:15, v/v) was used as the mobile phase. For isocratic elution, the flow rate of the mobile phase was maintained at 0.550 mL/min. The autosampler temperature was kept at 5 °C and the pressure of the system was 1400 psi. A triple quadrupole mass spectrometer MDS SCIEX API-5500 (Toronto, Canada) equipped with electrospray ionization and operated in negative ionization mode was used for detection and quantification of analytes and ISs. The optimized source parameters like ion spray voltage, turbo heater temperature, curtain gas, Gas1, Gas2, and collision activation dissociation were kept at –4500 V, 500 °C, 30 psi, 50 psi, 60 psi and 8, respectively. The compounded dependent parameters and multiple reaction monitoring (MRM) transitions for analytes and ISs are compiled in Table 1. Analyst classic software version 1.5.2 was used to control all parameters of LC and MS/MS.

2.3. Calibrators and quality control samples

Calibration standards (CSs) were made at 0.50, 1.00, 3.00, 6.00,

12.0, 25.0, 75.0, 125.0, and 250.0 ng/mL concentrations for both the analytes. The quality control (QC) samples were prepared at five levels, viz. 200.0 ng/mL (high quality control, HQC), 100.0 and 20.0 ng/mL (medium quality control MQC-1 and 2), 1.50 ng/mL (low quality control, LQC) and 0.50 ng/mL (lower limit of quantification quality control, LLOQ QC) for LIS and HCTZ, respectively.

2.4. Protocol for sample preparation

Prior to extraction, all frozen subject samples, CSs and QC samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 μL spiked plasma/subject sample, 50 μL of combined IS solution was added and vortexed for 30 s. Further, 100 μL of 5.0 mM ammonium formate (pH 3.0) was added and vortex mixed for another 30 s. Thereafter, the samples were centrifuged at 14,000 g for 5 min at 10 °C. Prior to sample loading, solid phase extraction (SPE) cartridges were conditioned by passing 1.0 mL of methanol followed by 1.0 mL of 5.0 mM ammonium formate (pH 3.0). The plasma matrix was drained out from the extraction cartridges by applying positive nitrogen pressure. The samples were washed with 1.0 mL of 5.0 mM ammonium formate solution, followed by 1 mL of water. Drying of cartridges was done for 1.0 min by applying nitrogen (1.72×10⁵ Pa) at 2.4 L/min flow rate. The samples were eluted with 0.5 mL mobile phase solution into pre-labeled vials, briefly vortexed and 5.0 μL of the eluant was used for injection in the chromatographic system.

2.5. Pharmacokinetics and incurred sample reanalysis

The aim of the study was to determine the bioequivalence of a 10 mg LIS and 12.5 mg HCTZ FDC test formulation (Generic Company, India) with a corresponding reference formulation, ZESTORETIC® (10 mg LIS+12.5 mg HCTZ) FDC tablets from AstraZeneca UK (Bedfordshire, UK). The design was an open label, balanced, randomized, two-treatment, two-period, two-sequence, single dose, crossover study in 18 healthy adult Indian subjects under fasting condition. All the subjects were informed of the aim and risk involved in the study and written consent was obtained. An Independent Ethics Committee approved the study protocol. The study was conducted in accordance with International Conference on Harmonization, E6 Good Clinical Practice Guidelines [33]. Health check-up for all subjects was done by general physical examination, electrocardiogram (ECG) and laboratory tests like hematology, biochemistry and urine examination. The subjects were orally administered with a single dose of test/reference formulation with 240 mL of water after a wash out period of 7 days. Blood samples were collected in vacutainers containing K₃EDTA at 0.00 (pre-dose), 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 12.0, 16.0, 24.0, 36.0, 48.0, 72.0 and 96.0 h of drug administration. Blood samples were centrifuged at 1811 g at 4 °C for 15 min; plasma was separated and stored at –70 °C until use. The pharmacokinetic parameters of LIS and HCTZ were estimated by non-compartmental model using WinNonlin software version 5.2.1 (Certara, Princeton, NJ 08540, USA).

Incurred sample reanalysis (ISR) is now an integral part of

Table 1

Compound dependent mass parameters for lisinopril, hydrochlorothiazide and their labeled internal standards.

Compounds	Q1 mass (amu)	Q3 mass (amu)	Dwell time (ms)	Declustering potential (V)	Entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)
Lisinopril	404.3	114.1	200	–85.0	–12.0	–30.0	–10.0
Lisinopril-d5	409.3	114.1	200	–85.0	–12.0	–25.0	–10.0
Hydrochlorothiazide	296.0	204.9	200	–95.0	–12.0	–33.0	–18.0
Hydrochlorothiazide-13C,d2	299.0	205.9	200	–95.0	–12.0	–31.0	–18.0

Quadrupole 1 and 2 were set at unit mass resolution

bioanalytical methodology for preclinical and clinical studies. The method reproducibility was assessed by reanalysis of 87 incurred samples near the C_{max} and the elimination phase in the pharmacokinetic profile of the drugs. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. As per the acceptance criterion at least two-thirds of the original results and repeat results should be within 20% of each other [34].

3. Results and discussion

3.1. LC-ESI-MS/MS method development

Several reports have used positive ionization mode for LC-MS/MS analysis of LIS [12,13], while HCTZ which has polar groups gives a good mass spectrometric response in the negative mode [26,28]. As LIS is a polyfunctional, ampholyte molecule containing two basic and two acidic moieties (pK_a 10.75, 7.13, 3.13 and 1.63), full scan mass spectra was acquired for both the analytes in the positive and negative ionization modes for electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) by direct infusion of 100 ng/mL solution of the analytes. As ESI provided better ionization efficiency than APCI, it was selected in the present work. Further, it was observed that HCTZ gave much higher response in the negative mode while the intensity was comparable in both the modes for LIS. Thus, negative ionization mode was selected in the present work as it showed better selectivity without compromising the sensitivity for LIS. Moreover, switching of polarities requires some time for stabilization and may therefore lead to noisy baseline. The observed Q1 MS spectra in the negative mode showed predominant deprotonated molecular ions $[M-H]^-$ at m/z 404.3, 409.3, 296.0 and 299.0 for LIS, LIS-d5, HCTZ and HCTZ-13C,d2, respectively. The collision induced dissociation of $[M-H]^-$ ions gave intense fragment/product ion at m/z 114.1 for LIS and LIS-d5 (ascribed to pyrrolidine-2- carboxylic acid) and m/z 204.9 for HCTZ and m/z 205.9 for HCTZ-13C,d2 (formed due to elimination of HCN and NH_3 from the deprotonated molecular ion) as shown in Fig. 1. Additionally, qualifier transitions of m/z 289.2 and 268.9 were also monitored for unambiguous identification of LIS and HCTZ,

respectively. A dwell time of 200 ms was sufficient and no cross talk was observed between the MRMs of LIS and LIS-d5 having identical product ions.

To optimize the LC conditions, several reversed phase columns having different dimensions were tested, ACE C_{18} (50/100 mm×4.6 mm, 5.0 μ m), Gemini C_{18} (50/100 mm×4.6 mm, 5.0 μ m), Cosmosil C_{18} (50/100 mm×4.6 mm, 5.0 μ m) and Hypersil Gold C_{18} (50/100 mm×3.0 mm, 5 μ m). For chromatographic separation of these drugs various combinations of acetonitrile/ methanol and acidic buffers (ammonium formate/ammonium acetate) in the pH range of 3.0–5.5 were attempted in order to obtain symmetrical peak shapes, suitable retention and adequate signal-to-noise ratio leading to lower limits of quantitation. It was observed that the mobile phase composition and pH played a major role in chromatographic separation of these drugs as LIS has acidic as well as basic groups while HCTZ has pK_a of 7.9 and 9.2 [28]. Compared to methanol, acetonitrile helped in providing higher sensitivity and sharp peaks shapes. Another important observation was that higher proportion (> 75%) of organic diluents was necessary for optimum resolution of the drugs. In addition, control of pH was necessary especially for HCTZ as it directly affected its retention, while the response for both the drugs was much higher with ammonium formate compared to ammonium acetate buffer. Although the peaks were satisfactorily resolved ($R_s \geq 1.5$) on all four columns using acetonitrile and 5.0 mM ammonium formate (pH 4.5 adjusted with 0.1% formic acid) (85:15, v/v) as the mobile phase, the response was not adequate on Gemini C_{18} , and the peak shapes were not acceptable with ACE C_{18} and Cosmosil C_{18} columns. Nevertheless, the best chromatographic conditions were achieved on Hypersil Gold C_{18} column with adequate response, resolution ($R_s \geq 3.7$), symmetric peak shape, baseline separation within 2.0 min (Fig. 2). The retention time of LIS, HCTZ, LIS-d5 and HCTZ-13C,d2 were found to be 1.44, 0.83, 1.43 and 0.84 min, respectively. The capacity factor (k), which shows the relative rates of migration of the analytes on the column, was 2.01 and 0.73 for LIS and HCTZ, respectively. Further, use of labeled internal standards which had identical chromatographic behavior helped to achieve acceptable method performance.

Literature presents use of all three conventional extraction techni-

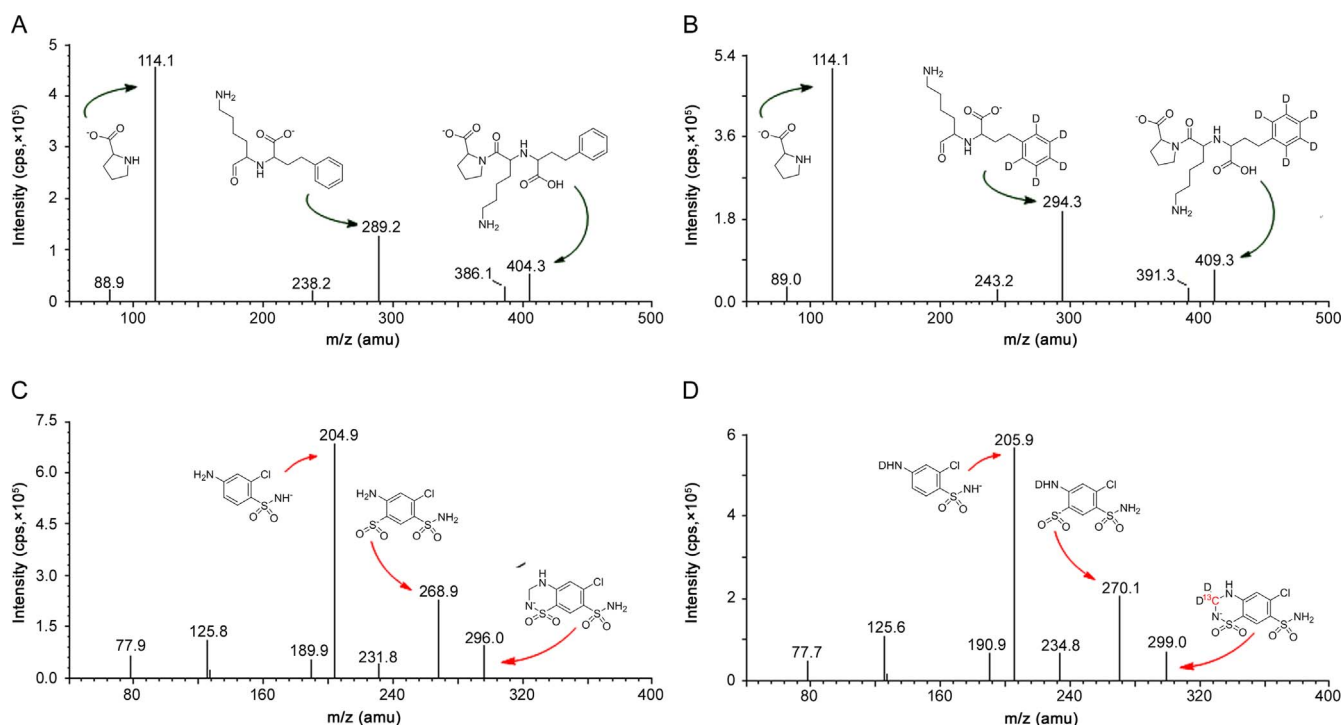


Fig. 1. Product ion mass spectra of (A) lisinopril (m/z 404.3→114.1), (B) lisinopril-d5, IS (m/z 409.3→114.1), (C) hydrochlorothiazide (m/z 296.0→204.9), and (D) hydrochlorothiazide-13C, d2, IS (m/z 299.0→205.9) in the negative ionization mode.

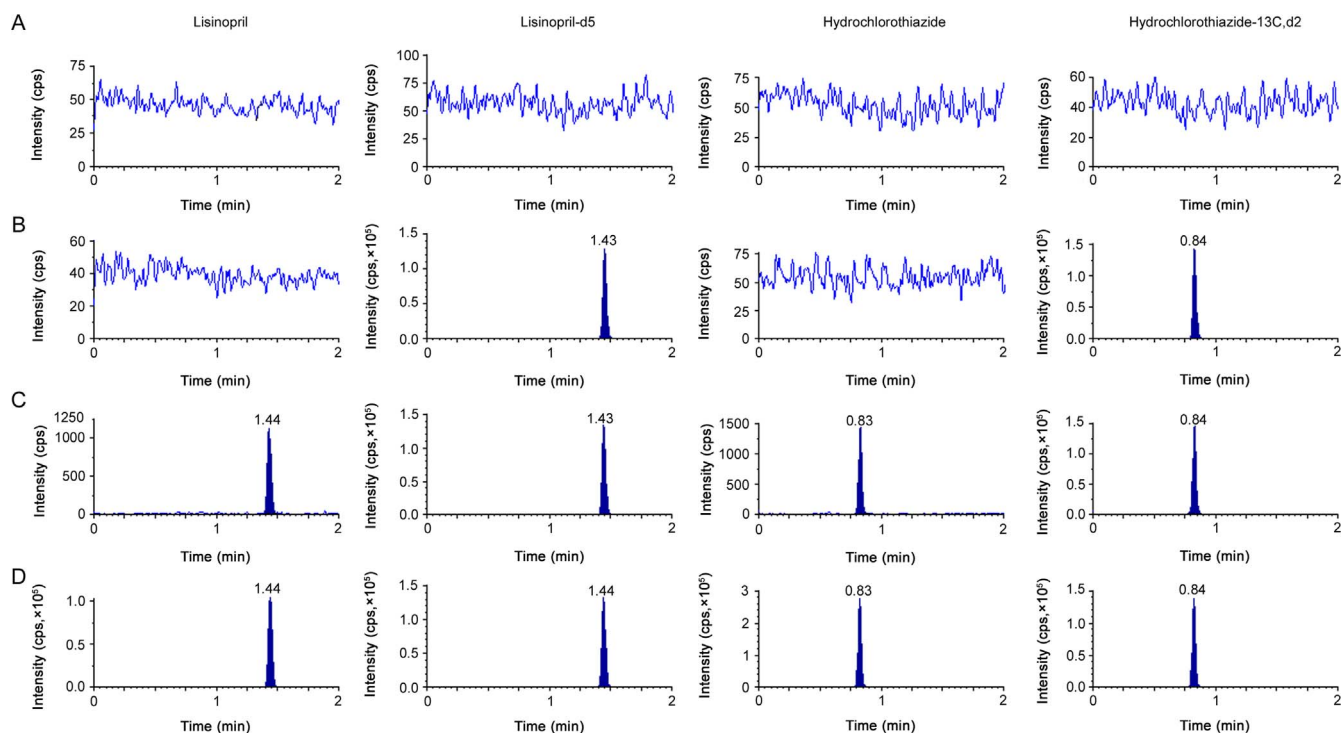


Fig. 2. MRM ion-chromatograms of lisinopril, lisinopril-d5, hydrochlorothiazide and hydrochlorothiazide-13C, d2 in (A) double blank plasma, (B) blank plasma spiked with IS, (C) at 0.50 ng/mL concentration of analyte with IS, and (D) real subject sample at C_{\max} after oral administration of 10 mg lisinopril and 12.5 mg hydrochlorothiazide tablet formulation.

Table 2

Intra-batch and inter-batch precision and accuracy for lisinopril and hydrochlorothiazide.

Nominal concentration (ng/mL)	Intra-batch (n=6)			Inter-batch (n=30; 6 from each batch)		
	Mean conc. found (ng/mL)	CV (%)	Accuracy (%)	Mean conc. found (ng/mL)	CV (%)	Accuracy (%)
Lisinopril						
200.0	198.8	2.03	99.4	200.4	0.69	100.2
100.0	98.90	1.33	98.9	101.3	1.04	101.3
20.00	20.34	2.55	101.7	19.92	1.92	99.6
1.500	1.508	3.83	100.5	1.534	2.71	102.3
0.500	0.488	4.04	97.6	0.495	4.84	99.0
Hydrochlorothiazide						
200.0	200.6	3.39	100.3	197.6	1.37	98.8
100.0	96.24	2.66	96.2	100.4	0.91	100.4
20.00	20.64	1.02	103.2	19.94	1.83	99.7
1.500	1.480	3.45	98.7	1.540	4.57	102.7
0.500	0.499	5.26	99.8	0.506	3.02	101.2

CV: Coefficient of variation.

ques, protein precipitation (PP), liquid-liquid extraction (LLE) and SPE for the separate analysis of LIS [10–12] and HCTZ [17–19] in human plasma. One report for the simultaneous determination of LIS along with other ACE inhibitors and HCTZ has established PP with acetonitrile as well as SPE using 500 μ L plasma or urine [31]. However, the recovery for LIS using SPE was low (62.8%–70.3%), and HCTZ could not be determined by PP due to endogenous matrix components and the limit of quantitation was 120 and 56 ng/mL for LIS and HCTZ, respectively. However, based on our previous work with HCTZ [28], SPE was used for sample preparation on Oasis HLB cartridges. Use of 5.0 mM ammonium formate (pH 3.0) was essential for preconditioning of the cartridges and also during washing step. Elution with mobile phase solution helped in obtaining quantitative and consistent recovery for LIS (97.3%–101.4%) and HCTZ (96.6%–103.1%) from 100 μ L plasma sample.

3.2. Method validation results

The method was validated in accordance with the United States Food and Drug Administration (USFDA) guidance [35] and the procedures followed were similar to our previous method [36].

The column and autosampler carryover that might influence the accuracy and precision of the method was evaluated by injecting extracted double blank plasma sample following the highest CS concentration (250 ng/mL). The results showed no significant carryover ($\leq 0.60\%$ of LLOQ) or contamination for both the analytes.

All five calibration curves established over the validated concentration range of 0.50–250.0 ng/mL gave good linearity with the correlation coefficient value, $r^2 \geq 0.9996$ for LIS and HCTZ. The mean linear equations calculated for LIS and HCTZ were $y = (0.00978 \pm 0.00011) x - (0.00001 \pm 0.00026)$ and $y = (0.01018 \pm 0.00008) x - (0.00023 \pm 0.00028)$, respectively. The accuracy and precision (% CV) for the

Table 3
Extraction recovery and matrix factor for lisinopril and hydrochlorothiazide.

Quality control level (ng/mL)	Mean area response (n=6)			Recovery (B/A %)		Matrix factor		
	A (post-extraction spiking)	B (pre-extraction spiking)	C (neat samples in mobile phase)	Analyte	IS	Analyte (A/C)	IS	IS-normalized (Analyte/IS)
Lisinopril								
200.0	3,362,465	3,409,540	3,466,459	101.4	101.7	0.97	0.95	1.02
100.0	1,659,228	1,624,384	1,642,800	97.9	98.4	1.01	1.02	0.99
20.00	329,707	324,762	323,242	98.5	97.2	1.02	0.99	1.03
1.500	24,833	24,163	25,339	97.3	95.8	0.98	1.01	0.97
Hydrochlorothiazide								
200.0	4,109,656	4,105,547	4,325,953	99.9	97.5	0.95	0.97	0.98
100.0	2,140,559	2,067,780	2,162,181	96.6	95.1	0.99	0.97	1.02
20.00	418,769	409,556	436,217	97.8	98.2	0.96	0.99	0.97
1.500	30,575	31,523	29,399	103.1	101.8	1.04	1.01	1.03

IS: internal standard.

Table 4
Stability of lisinopril and hydrochlorothiazide in plasma under different conditions (n=6).

Storage conditions	Nominal concentration (ng/mL)	Lisinopril		Hydrochlorothiazide	
		Mean stability sample (mean ± SD, ng/mL)	Change (%)	Mean stability sample (mean ± SD, ng/mL)	Change (%)
Bench top stability at 25 °C, 18 h	200.0	203.6 ± 5.5	1.81	202.2 ± 4.9	1.08
	1.50	1.49 ± 0.05	-0.39	1.52 ± 0.05	1.13
Freeze-thaw stability at -20 °C	200.0	200.4 ± 4.2	2.11	197.2 ± 2.8	-1.40
	1.50	1.52 ± 0.04	1.57	1.42 ± 0.07	-5.32
Freeze-thaw stability at -70 °C	200.0	203.9 ± 3.9	1.97	204.9 ± 3.8	2.45
	1.50	1.44 ± 0.05	-4.07	1.49 ± 0.06	-0.76
Processed sample stability at 25 °C, 32 h	200.0	194.4 ± 2.7	-2.81	196.7 ± 5.3	-1.63
	1.50	1.42 ± 0.06	-5.47	1.54 ± 0.04	2.48
Autosampler stability at 5 °C, 36 h	200.0	203.4 ± 5.8	1.68	202.8 ± 1.9	1.40
	1.50	1.55 ± 0.07	3.25	1.53 ± 0.06	1.64
Long term stability at -20 °C, 176 days	200.0	194.2 ± 1.4	-2.90	196.3 ± 6.8	-1.83
	1.50	1.54 ± 0.03	1.76	1.45 ± 0.03	-3.59
Long term stability at -70 °C, 176 days	200.0	203.0 ± 2.1	1.50	203.3 ± 3.8	1.64
	1.50	1.43 ± 0.01	-4.73	1.46 ± 0.05	-2.43

SD: Standard deviation.

$$\text{Change\%} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100.$$

CSs ranged from 98.0% to 102.2% and 0.93% to 3.42% for both the analytes respectively. The lowest concentration in the standard curve for the analytes was measured at a signal-to-noise ratio (S/N) of ≥ 18 . For intra-batch and inter-batch study, the results for precision (% CV) varied from 0.69% to 5.26%, while the accuracy ranged from 96.2% to 103.2% across QC samples (Table 2).

The selectivity of the method was evaluated from eight different plasma sources. The results showed no endogenous peaks that would interfere with the signal of the analytes in the blank plasma. The mean extraction recovery and IS-normalized matrix factors (MFs) for LIS and HCTZ are given in Table 3. The recovery obtained was highly consistent and quantitative for both the analytes. Assessment of matrix effect is essential as co-eluting matrix components can have a direct impact on the overall reliability of a validated method. The IS-normalized MFs calculated using labeled IS should be close to unity due to similarities in the physico-chemical properties and elution behavior of the analyte and IS. The IS-normalized MFs ranged from 0.97 to 1.03 for both the analytes. Further, it is required to check the relative matrix effect in lipemic and haemolysed plasma samples in addition to normal K₃EDTA plasma.

The stability of LIS and HCTZ was extensively evaluated in stock solutions and in plasma samples under different storage conditions. Stock/working solutions kept for short-term stability remained stable at room temperature up to 16 h, and for a minimum of 54 days at refrigerated temperature (5 °C) for long-term stability of the analytes and ISs. Bench-top stability at 25 °C, processed sample stability at 25 °C and autosampler stability at 5 °C were determined up to 18 h, 32 h and 36 h, respectively without significant loss of drugs. Analytes were found to be stable for a minimum of six freeze-thaw cycles, while the samples stored for assessment of long-term stability of the analytes in plasma were stable for at least 176 days. The results for different stability experiments performed in plasma are shown in Table 4.

3.3. Application of the method and ISR results

Presently there are no reports on the pharmacokinetics of LIS and HCTZ in Indian subjects for an FDC formulation. After oral administration of 10 mg LIS and 12.5 mg HCTZ FDC tablets to 18 healthy Indian volunteers, the plasma concentrations of LIS and HCTZ were determined for a bioequivalence study using the newly developed

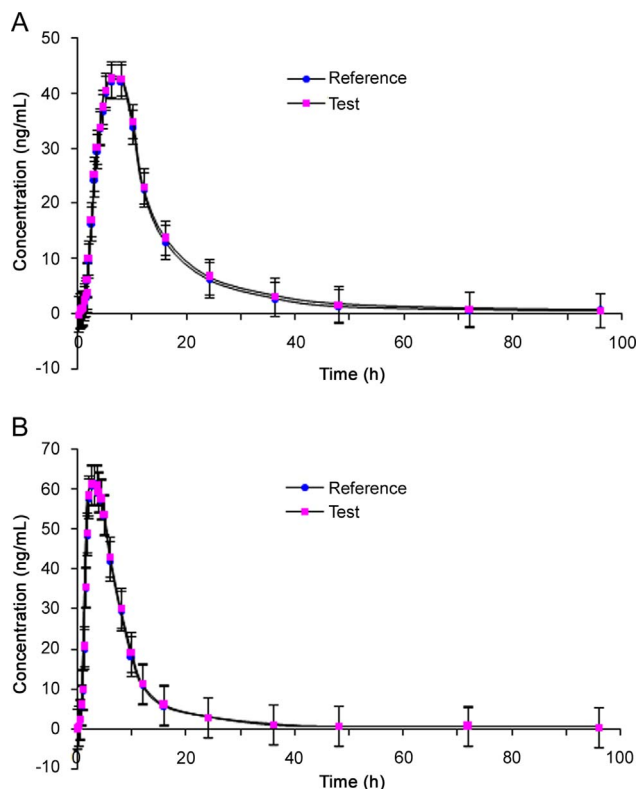


Fig. 3. Mean plasma concentration-time profiles of (A) lisinopril and (B) hydrochlorothiazide after oral administration of 10 mg lisinopril and 12.5 mg hydrochlorothiazide fixed dose tablet of test and reference formulation to 18 healthy Indian subjects.

Table 5

Mean pharmacokinetic parameters (\pm SD) following oral administration of 10 mg lisinopril and 12.5 mg hydrochlorothiazide combination formulation to 18 healthy Indian subjects under fasting condition.

Parameter	Lisinopril		Hydrochlorothiazide	
	Test	Reference	Test	Reference
C_{max} (ng/mL)	42.79 \pm 7.33	42.34 \pm 8.48	61.12 \pm 18.08	60.87 \pm 20.35
T_{max} (h)	8.20 \pm 0.60	7.95 \pm 0.45	2.55 \pm 0.18	2.70 \pm 0.29
$t_{1/2}$ (h)	12.03 \pm 0.52	11.89 \pm 0.31	9.49 \pm 0.80	9.91 \pm 0.58
AUC_{0-96} (h-ng/mL)	620.37 \pm 48.14	587.93 \pm 45.44	484.38 \pm 69.63	470.91 \pm 56.19
AUC_{0-inf} (h-ng/mL)	682.41 \pm 52.75	656.72 \pm 53.19	514.01 \pm 79.38	499.21 \pm 69.73
Kel (1/h)	0.057 \pm 0.021	0.0583 \pm 0.013	0.073 \pm 0.017	0.070 \pm 0.026

C_{max} : maximum plasma concentration; T_{max} : time point of maximum plasma concentration; $t_{1/2}$: half-life of drug elimination during the terminal phase; AUC_{0-t} : area under the plasma concentration-time curve from 0 h to 96 h; AUC_{0-inf} : area under the plasma concentration-time curve from 0 h to infinity; Kel: elimination rate constant; SD: standard deviation.

LC-MS/MS method. Fig. 3 shows the mean plasma concentration-time profiles for LIS and HCTZ under fasting condition. The mean values of pharmacokinetic parameters obtained after oral administration of test and reference formulation are summarized in Table 5. Though it was not possible to compare the results directly with a previous study in Chinese subjects due to different dose strengths [34], the T_{max} values obtained in our work were higher for LIS and HCTZ. Conversely, the $t_{1/2}$ was slightly less for LIS and comparable for HCTZ from their results. The reason for this variation can be related to genetic, physiological and pathological factors in different ethnic groups. However, the ratios of mean log-transformed parameters and their 90% confidence inter-

vals for C_{max} , $AUC_{0-96 h}$ and AUC_{0-inf} were within the acceptance range of 80%–125% (98.53%–111.70% for LIS and 95.80%–107.23% for HCTZ), which confirms that the test and reference formulations were pharmacokinetically equivalent in terms of rate and extent of drug absorption. Further, the assay reproducibility test performed with 87 incurred samples showed % change within \pm 14% of the initial analysis results, which is within the acceptance criteria of \pm 20% (Fig. S1).

4. Conclusions

To the best of our knowledge this is the first report for the simultaneous determination of LIS and HCTZ by LC-MS/MS in human plasma. The proposed LC-MS/MS method allows efficient extraction of LIS and HCTZ from 100 μ L plasma samples and provides higher sensitivity for their determination compared to reported procedures. The SPE procedure gave highly consistent and reproducible recoveries for both the analytes. The method permits simultaneous analysis of the analytes in 2.0 min, which is a significant improvement in comparison to existing methods. The use of negative ionization mode without polarity switch for mass detection provided additional selectivity to the method. The method was fully validated as per the current regulatory requirements and was successfully applied for a bioequivalence study of an FDC formulation of LIS and HCTZ in healthy subjects with good reproducibility.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2016.11.004.

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