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Simultaneous analysis of losartan, its active metabolite, and hydrochlorothiazide in human plasma by a UPLC-MS/MS method

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Abstract: A selective and sensitive ultra performance liquid chromatography-tandem mass spectrometry method was developed for the simultaneous determination of losartan (LOS), EXP-3174, which is an active metabolite LOS carboxylic acid, and hydrochlorothiazide (HCTZ) in human plasma. Solid-phase extraction was carried out on Oasis HLB cartridges with 100 μL of plasma to give an extraction recovery in the range of 88.5%–102.5% for the three analytes. Chromatography on a BEH C18 column afforded baseline separation of all the analytes within 2.4 min using 1.0% formic acid in water and acetonitrile (15:85, v/v) as the mobile phase. Quantitation was performed with multiple reaction monitoring in the negative ionization mode. The response of the method was linear over a dynamic range of 0.5–500, 1.0–750, and 0.25–150 ng/mL for LOS, EXP-3174, and HCTZ, respectively. Extent of signal suppression/enhancement was examined through postcolumn infusion. The effect of matrix components was evaluated by postextraction spiking and calculation of the slope of calibration lines. The method was successfully applied to a bioequivalence study of 50 mg losartan and 12.5 mg hydrochlorothiazide tablet formulation in 65 healthy human subjects. Reproducibility of the method was shown by reanalysis of 213 incurred samples.

Key words: Losartan, EXP-3174, hydrochlorothiazide, solid phase extraction, UPLC-MS/MS, human plasma

1. Introduction

Losartan (LOS) is a nonpeptide, orally active, and selective angiotensin II Type 1 (AT_1) receptor antagonist drug used mainly to treat hypertension associated with heart failure or renal impairment. It differs from angiotensin-converting enzyme (ACE) inhibitors by producing direct antagonism II receptors.[1,2] LOS is well absorbed following oral administration with an oral bioavailability of about 33% and reaches peak serum levels in 1.0 h. It undergoes significant first-pass metabolism to produce an active 5-carboxylic acid metabolite, designated as EXP-3174, which is mediated by cytochrome P450 enzymes CYP3A4 and CYP2C9. This metabolite is a long-acting (up to 24 h), noncompetitive antagonist at the AT_1 receptor and contributes to the pharmacological effects of LOS. It is 10–40 times more potent in blocking AT_1 receptors than the parent drug.[3] Hydrochlorothiazide (HCTZ) is a popular diuretic of the thiazide class that reduces plasma volume by increasing the excretion of sodium, chloride, and water. The decrease in plasma volume results in counter-regulatory stimulation of the rennin-angiotensin system and the sympathetic nervous system.[4] Thus, the complimentary action of an angiotensin II receptor antagonist and a thiazide has led to their extensive use in the treatment of

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patients with overt heart failure. Clinical studies have demonstrated antihypertensive efficacy and tolerability of LOS-HCTZ combination therapy in patients with moderate-to-severe essential hypertension, which is otherwise inadequately controlled by monodrug therapy.[5,6] Thus, with the advancement of medical science, there is an immense role of combination therapy with multiple drugs or at least two drugs having different modes of action in the treatment of hypertension.[7]

Several methods are reported for the quantitation of LOS, alone,[8–10] in the presence of its active metabolite, EXP-3174,[11–18] with other angiotensin II receptor antagonists,[19,20] and also with other classes of drugs in binary,[21,22] ternary[23,24] and quaternary[25] combinations in different biological matrices. Similarly, a number of bioanalytical methods are presented for the determination of HCTZ as a single analyte,[26–29] and in the presence of different antihypertensive drugs in binary[30–40] and ternary[41,42] combinations in various biological fluids including human plasma,[26–33,36–42] human urine,[32,34] and rat plasma.[35]

So far few methods are reported for the simultaneous analysis of LOS, EXP-3174, and HCTZ.[7,43–47] Amongst these methods some have reported determination of LOS and HCTZ,[43,44] while the rest deal with the analysis of EXP-3174 along with LOS and HCTZ.[7,45–47] Kolocouri et al.[45] have reported a LC-MS/MS method for the simultaneous determination of all three analytes in human plasma. However, the total analysis time was substantially high, involving a lengthy sample preparation protocol through a fully automated 96-well-format–based solid phase extraction. Moreover, the calibration range set for the analytes is narrow and the method is not adequately sensitive for pharmacokinetic applications. Two other methods describe separate procedures for sample preparation and chromatographic separation of LOS, EXP-3174, and HCTZ in human plasma.[46,47] Both these methods have low sensitivity and long analysis time (5–10 min). Goswami et al.[7] have presented a promising LC-MS/MS method but the analytes (LOS and EXP-3174) were not chromatographically resolved, three separate internal standards were used for each of the analyte, and a large plasma volume was employed (500 μL) for sample preparation.

So far there are no methods based on UPLC-MS/MS for the simultaneous determination of LOS, EXP-3174, and HCTZ in human plasma. Thus, the objective of this work was to develop and fully validate a selective, rapid, and adequately sensitive method for the simultaneous estimation of all three analytes. The method presents an efficient solid-phase extraction of the analytes with quantitative recovery. The total analysis time (extraction and chromatography) was approximately 10 min. Additionally, the method presented has higher sensitivity and employs a much lower plasma sample for processing compared to all other reported methods. The method was successfully applied to a bioequivalence study of 50 mg losartan potassium and 12.5 mg hydrochlorothiazide hydrochloride fixed dose tablet formulation in 65 healthy human subjects. Further, the reproducibility of the method was suitably demonstrated by reanalysis of 213 incurred samples.

2. Results and discussion

2.1. Method development

Selective determination of LOS, EXP-3174, and HCTZ in human plasma is difficult due to their different physicochemical properties. All three analytes are weak acids with pKa of 5.6 and 5.4 for LOS and EXP-3174, respectively, corresponding to the acidic nitrogen protons in the tetrazole ring, and another pKa of 4.2 for EXP-3174 due to the carboxy group.[11] Similarly, HCTZ has pKa values of 7.9 and 9.2 due to the secondary amine and sulfonamide group, respectively.[43] Due to the significant difference in pKa values it was imperative to set optimum conditions for plasma extraction, chromatography, and mass detection for their simultaneous

determination. Mass spectrometric detection was preferred compared to UV,[8,9,11,21,25,26,31,36] diode array,[34,35] or fluorescence detection,[15,22,23] to attain the desired sensitivity and selectivity of the method. For quantitation, earlier reports have used negative polarity for LOS, EXP-3174, and HCTZ[7,44,45] to achieve adequate response for their simultaneous analysis. Moreover, negative ionization mode is selective and highly sensitive for compounds with high electron affinity. Thus, negative ionization mode was selected to fragment the analytes and to obtain intense and consistent product ions. The deprotonated precursor ions $[M - H]^-$ at m/z 421.2, 435.2, and 295.9 were observed in Q1 MS for LOS, EXP-3174, and HCTZ, respectively. Characteristic product ions found in Q3

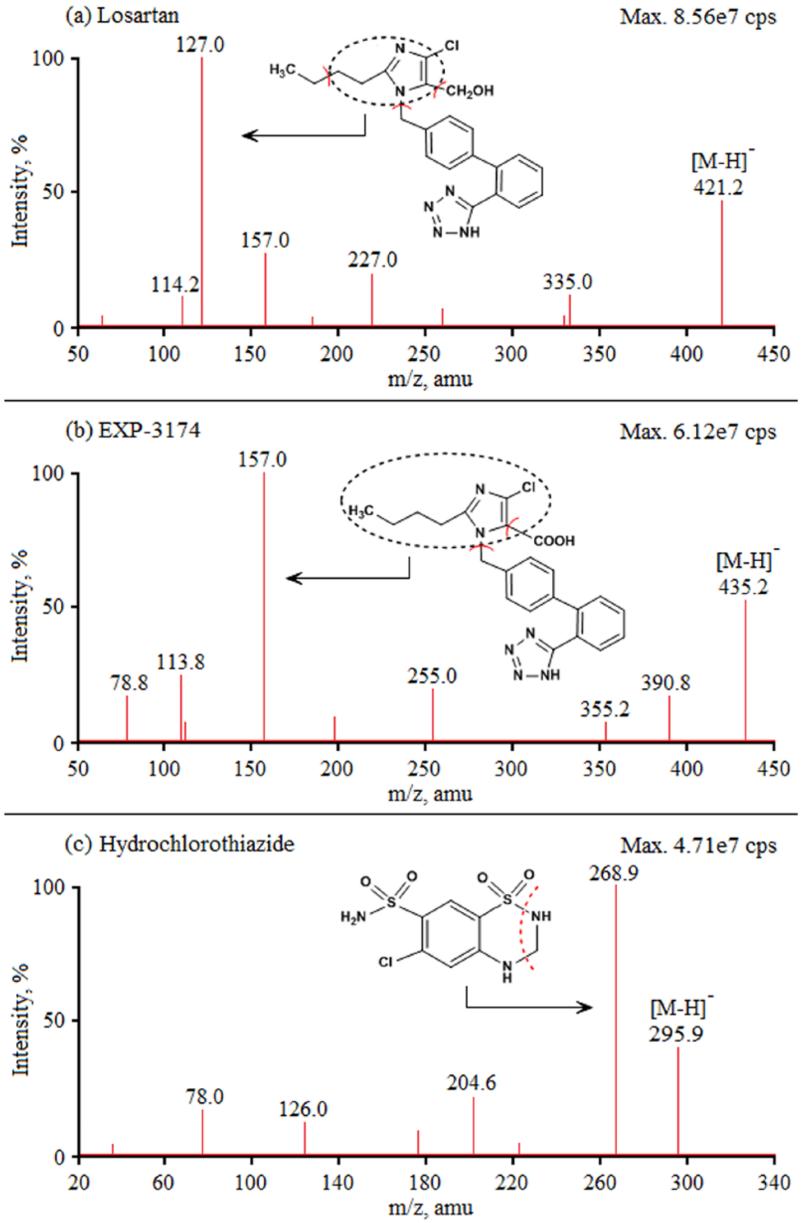

Figure 1. Product ion mass spectra of (a) losartan (m/z 421.2 → 127.0, scan range 50–450 amu), (b) EXP-3174 (m/z 435.2 → 157.0, scan range 50–450 amu) and (c) hydrochlorothiazide (m/z 295.9 → 268.9, scan range 20–340 amu) in negative ionization mode.

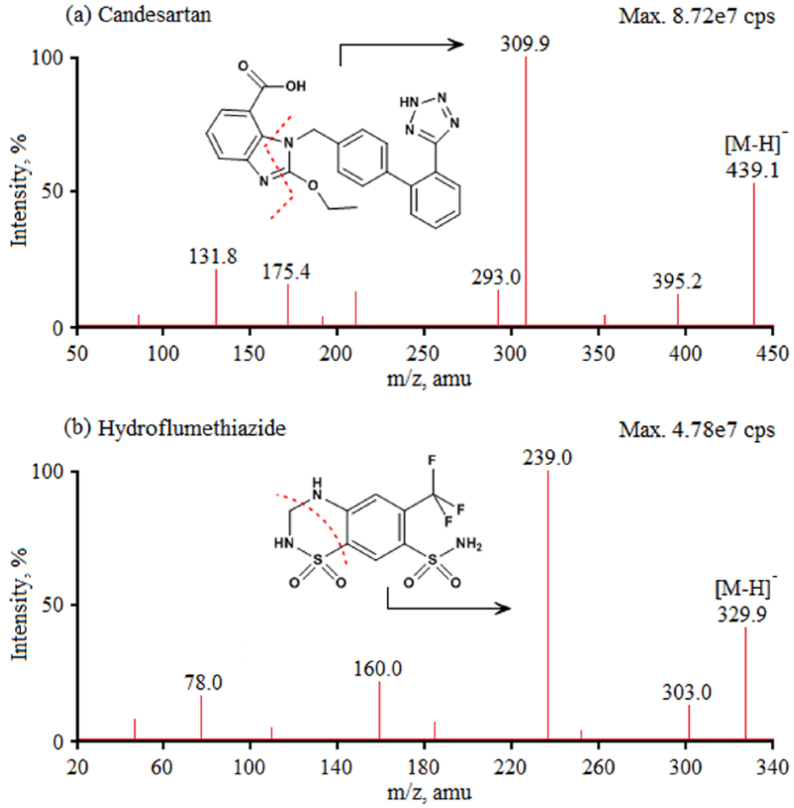

Figure 2. Product ion mass spectra of (a) candesartan (m/z 439.1 → 309.9, scan range 50–450 amu) and (b) hydroflumethiazide (m/z 329.9 → 239.0, scan range 20–340 amu) in negative ionization mode.

MS were at m/z 335.0, 227.0, 157.0, and 127.0 for LOS; m/z 390.8, 355.2, 255.0, and 157.0 for EXP-3174; and m/z 268.9, 204.6, 126.0, and 78.0 for HCTZ, respectively. However, the most stable and consistent fragment ions selected were m/z 127.0 and 157.0 for LOS and EXP-3174, respectively, having the imidazole ring (Figures 1a and 1b). For HCTZ, the major product ion was found at m/z 268.9 due to elimination of neutral species (HCN) from the precursor ion (Figure 1c). For the internal standard candesartan (CAN), the fragment at m/z 309.9 possessing the tetrazole ring (Figure 2a) and at m/z 239.0 (due to breaking up of benzthiazide ring) for hydroflumethiazide (HFMZ) (Figure 2b) were selected as the most abundant ions.

The chromatographic elution of the analytes on a Waters Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm) column was initiated as a rapid, sensitive, and rugged analytical method covering the dynamic linear range. The selection of mobile phase was crucial for synchronized determination of all the drugs having different pKa values. Thus, the pH of the mobile phase, buffer concentration, and choice and proportion of diluents were very important for chromatographic resolution with adequate response to achieve the desired sensitivity. Initially, acetonitrile/methanol with 10 mM ammonium acetate buffer (pH 6.5) gave higher response for LOS, EXP-3174, and CAN; however, the response for HCTZ and HFMZ was not reproducible. The signal was severely compromised at lower limit of quantitation (LLOQ) levels even after altering the concentration of buffer from 10 mM to 1.0 mM. Further, the chromatography was better with a higher response using an acetonitrile-buffer as compared to a methanol-buffer combination. Moreover, lowering the acetonitrile content in the mobile phase resulted in an increase in the retention of LOS and thereby the analysis time. Subsequent efforts were directed to optimize the pH of the mobile phase and the concentration of the buffer solution as they had significant

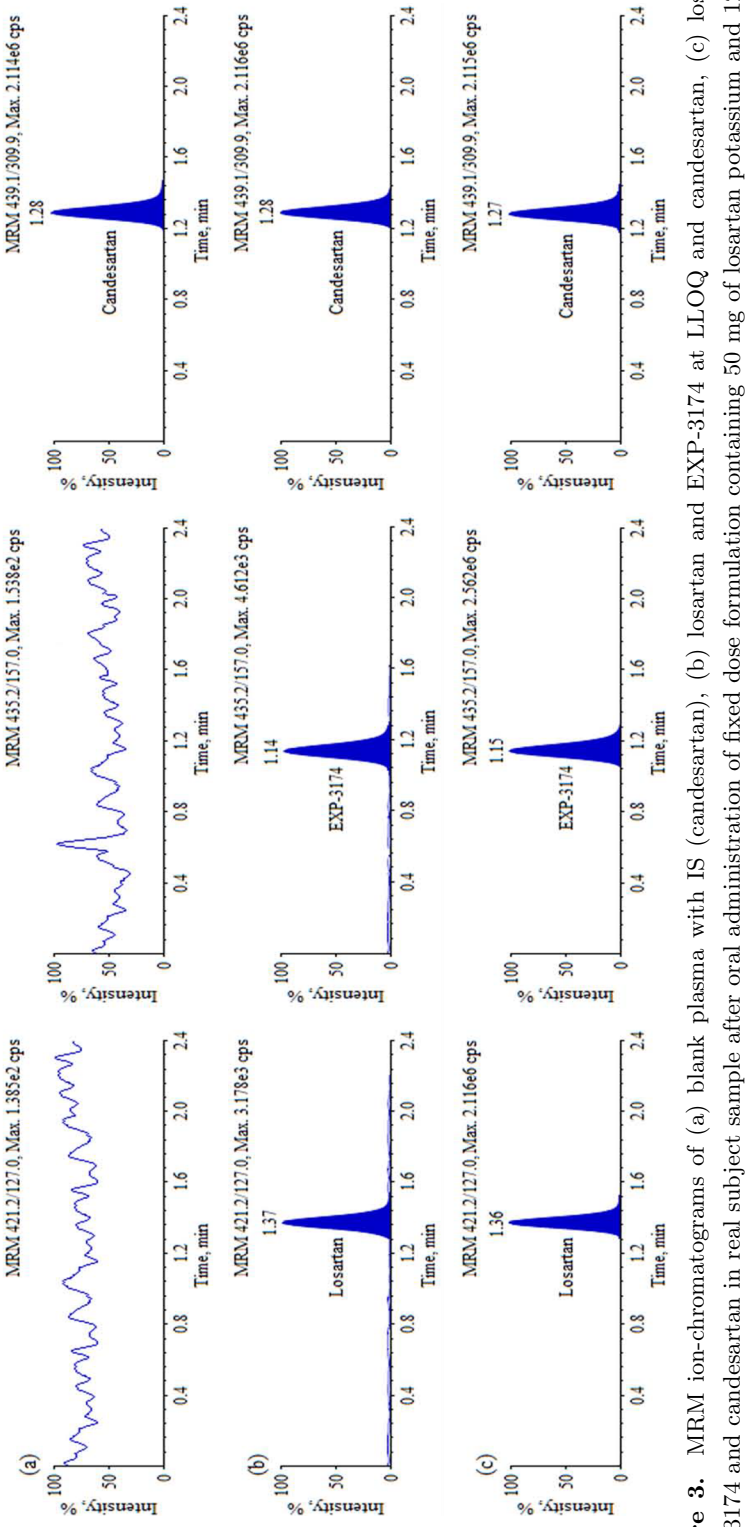

Figure 3. MRM ion-chromatograms of (a) blank plasma with IS (candesartan), (b) losartan and EXP-3174 at LLOQ and candesartan, (c) losartan, EXP-3174 and candesartan in real subject sample after oral administration of fixed dose formulation containing 50 mg of losartan potassium and 12.5 mg of hydrochlorothiazide hydrochloride.

impact on analyte retention, peak shape, and resolution. At pH above 5.0 the resolution between LOS and HCTZ was affected, which further deteriorated with increase in pH. Thus, to achieve greater reproducibility and better chromatography, low pH buffers were tried. Better reproducibility and peak shape were observed in acetonitrile:ammonium formate buffer, having pH 3.5 in 80:20 (v/v) ratio, but the signal to noise ratio for HCTZ was not adequate at LLOQ level. Finally, a superior signal to noise ratio ($\geq$22) and baseline resolution were obtained for all the analytes by replacing formate buffer with 1.0% (v/v) formic acid together with acetonitrile (15:85, v/v) having apparent pH 3.2 at a flow rate of 0.350 mL/min. There were no additional peaks due to endogenous plasma components as observed in one report when short columns (50 mm) were used even under MRM mode.[45] The chromatographic elution time for LOS, EXP-3174, HCTZ, CAN, and HFMZ was 1.37, 1.14, 1.87, 1.28, and 1.82 min, respectively, in a run time of 2.4 min (Figures 3a–3c and 4a–4c). The area ratio of analyte/internal standard was consistent for at least 100 injections at five QC levels. The salient chromatographic parameters like capacity factor and number of theoretical plates are presented in Table 1. The resolution factor between LOS and EXP-3174, LOS and HCTZ, and EXP-3174 and HCTZ was 1.01, 2.08, and 3.04, respectively. Ideally, a stable isotopically labeled analogue is preferred as an IS to account for any changes in ionization efficiency, solvent evaporation, and for overall performance of the method. In the present work general ISs were used that had structural similarity and belonged to the same class of drugs. CAN was used for LOS and EXP-3174, while HFMZ was used to monitor HCTZ. Unlike a previous report employing three separate ISs for the three analytes,[7] during method development trials it was evident that two ISs can effectively compensate any variability for improved accuracy and precision of the results.

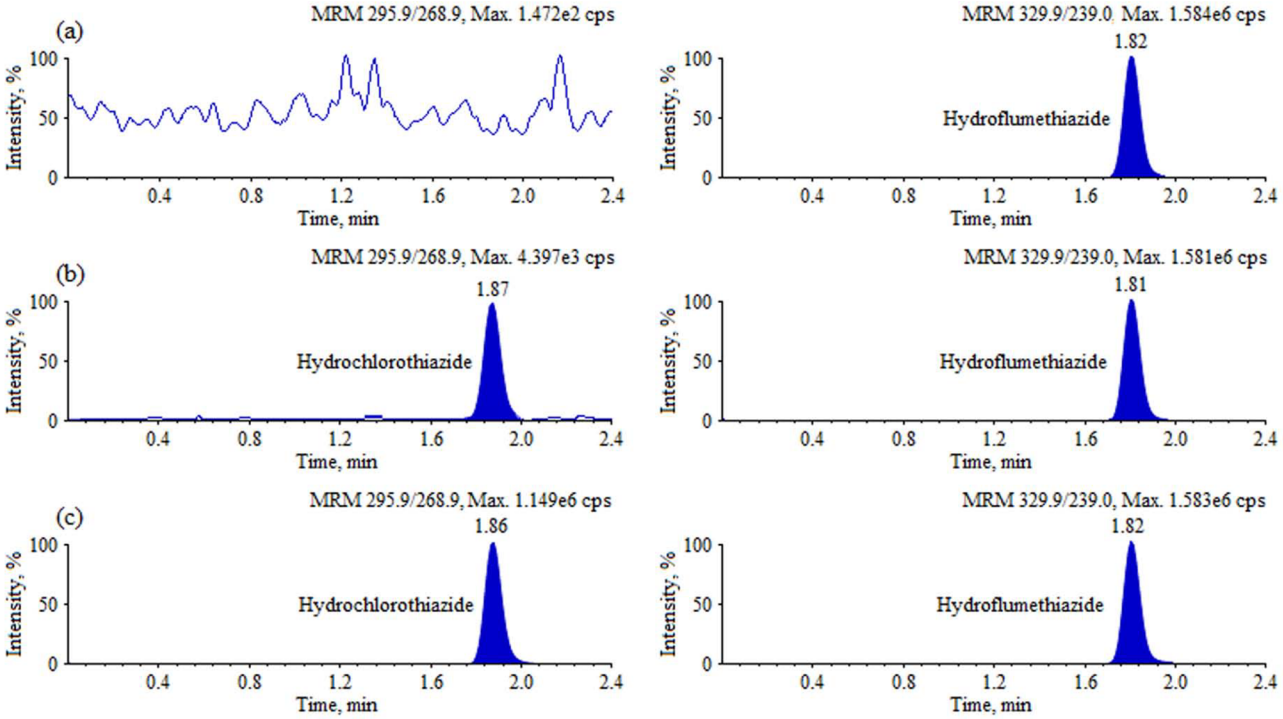

Figure 4. MRM ion-chromatograms of (a) blank plasma with IS (hydroflumethiazide), (b) hydrochlorothiazide at LLOQ and hydroflumethiazide, (c) hydrochlorothiazide and hydroflumethiazide in real subject sample after oral administration of fixed dose formulation containing 50 mg of losartan potassium and 12.5 mg of hydrochlorothiazide hydrochloride.

Table 1. Optimized mass spectrometer parameters, MRM transitions, and chromatographic performance.

Parameters	LOS	EXP-3174	HCTZ	CAN	HFMZ
Mass spectrometry parameters					
Source dependent					
Mode of analysis	Negative ionization				
Capillary voltage (kV)	3.94				
Cone voltage (V)	32				
Extractor voltage (V)	5.0				
RF lens (V)	0.0				
Source temperature (°C)	110				
Desolvation temperature (°C)	500				
Desolvation gas flow (L/h)	700 ± 10				
Cone gas flow (L/h)	150 ± 10				
Quadrupole 1 and 3	Unit mass resolution				
Analyzer parameters					
LM 1/HM 1 resolution	15.0/15.0				
Ion energy 1/ Ion energy 2	0.2/1.0				
Entrance/Exit	−1.0/0.1				
LM 2/ HM 2 resolution	14.0/14.0				
Compound dependent					
Cone voltage (V)	30	29	31	27	30
Collision energy (eV)	31	42	21	17	20
Dwell time (ms)	200	200	200	200	200
MRM transition (*m/z*)	421.2/127.0	435.2/157.0	295.9/268.8	439.1/309.9	329.9/239.0
Chromatography characteristics					
Retention time (min)	1.37	1.14	1.87	1.28	1.81
Capacity factors	2.04	1.53	3.15	1.84	3.02
Theoretical plates	521	361	971	455	909

LOS: losartan; EXP-3174: losartan carboxylic acid; HCTZ: hydrochlorothiazide; CAN: candesartan; HFMZ: hydroflumethiazide; RF: radio frequency; LM: low mass; HM: high mass

Sample preparation is an area of concern with respect to the volume of biological sample used and for high throughput analysis, especially with regard to the number of samples generated during clinical studies. Kolocouri et al.[45] used an automated multiprobe work station for liquid transfer steps during 96-well–based SPE after precipitation of plasma proteins with acetonitrile using 200 μL plasma samples. Although this method is well suited for high throughput applications, this facility may not be available in all labs. On the other hand, the method reported by Goswami et al.[7] offers a simplified off-line SPE procedure but employs large plasma volume (500 μL) for sample processing. Thus, to overcome these limitations and at the same time to achieve higher sensitivity for the analytes we modified the reported procedure.[7] As all three analytes have high protein binding ($\geq$68%), the plasma proteins were precipitated with 5% (v/v) *ortho*-phosphoric acid instead of acetonitrile[45] and use of ammonia solution[7] before loading the sample on the HLB cartridge. Further, the

Table 2. Salient features of methods developed for the simultaneous determination of LOS, EXP-3174, and HCTZ in human plasma.

Sr. No.	Detection technique; Column; mobile phase	Plasma volume, Extraction technique; Recovery	Linear range (ng/mL); Run time	Matrix effect	Application	Ref.
1	LC-MS/MS; Nucleosil S-3 µm, 100 Å (150 × 4.6 mm); 78% ACN and 22% 5.0 mM AA (v/v)	200 µL; 96-well-format–based SPE; NA	LOS & EXP-3174: 1.00–400, HCTZ: 0.500–200; 4.0 min	NA	NA	45
2	LC-MS/MS; Zorbax SBC18 (4.6 × 50 mm, 5 µm); MeOH:water, 5.0 mM AF & 0.02%–0.05% FA under gradient condition	750–1000 µL; SPE with MCX Oasis plates; NA	LOS: 2.02–807.20, EXP-3174: 2.51–1002.49, HCTZ: 1.01–150.80; NA	NA	Bioequivalence study of single dose of 100 mg LOS + 12.5 mg HCTZ combination tablet in 73 healthy subjects	46
3	LC-MS/MS; Discovery C18 (4.6 × 50 mm, 5 µm); ACN - AF buffer (pH 6.2 ± 0.5; 2 ± 0.1 mM) (90:10, v/v)	500 µL; SPE under alkaline condition of ammonia with Oasis HLB extraction cartridges; >80% for all three analytes	LOS: 2.54–1509.56, EXP-3174: 3.27–1946.38, HCTZ: 2.10–410.40; 3.0 min	NA	Bioequivalence study of single dose of 100 mg LOS + 25 mg HCTZ combination tablet in 60 healthy male subjects	7
4*	LC-MS/MS; Inertsil ODS-3 (150 × 4.6 mm, 5 µm) for LOS & EXP-3174, Venusil MP C18 (250 × 4.6 mm, 5 µm) for HCTZ; 0.1% FA- MeOH (27:73 v/v, pH 2.7) for LOS, 0.1% FA - MeOH (30:70 v/v, pH 2.7) for EXP-3174, water- MeOH (60:40, v/v) for HCTZ	LOS: 200 µL, EXP-3174: 500 µL, HCTZ: 200 µL; PP for LOS, LLE for EXP-3174 & HCTZ; NA	LOS: 5–800, EXP-3174: 5–750, HCTZ: 1–150; 10.0 min	96%–108%	Bioequivalence study of single dose of 50 mg LOS + 12.5 mg HCTZ combination tablet in 40 healthy male volunteers	47
5	UPLC-MS/MS; BEH C18 (50 × 2.1 mm, 1.7 µm); 1.0% (v/v) FA & ACN (15:85, v/v)	100 µL, SPE under acidic conditions of *o*-phosphoric acid with Oasis HLB extraction cartridges; 88.5–102.5 for all three analytes	LOS: 0.5–500, EXP-3174: 1–750, HCTZ: 0.25–150; 2.4 min	MF: 0.939–1.050; postcolumn infusion study	Bioequivalence study of single dose of 50 mg LOS + 12.5 mg HCTZ combination tablet in 65 healthy subjects; Incurred sample reanalysis with 213 study samples	PM

LOS: losartan; EXP-3174: losartan carboxylic acid; HCTZ: hydrochlorothiazide; PP: protein precipitation; LLE: liquid-liquid extraction; SPE: solid phase extraction; ACN: acetonitrile; MeOH: methanol; AA: ammonium acetate; FA: formic acid; AF: ammonium formate; MF: matrix factor; PM: present method; NA: data not available.

*Separate method for LOS, EXP-3174, and HCTZ

washing and elution steps were critically optimized without compromising the reproducibility and the recovery of all three analytes. Sequential use of 1.0 mL of 5% methanol in water and 1.0 mL of 5 mM ammonium formate ensured maximum removal of plasma components. Additionally, elution of analytes and ISs from the cartridge was carried out with 900 μL of acetonitrile:water (90:10, v/v) for optimum recovery in the range of 88.5%–102.5% for LOS, EXP-3174, and HCTZ. The salient features of the present method are compared with those of methods developed for the simultaneous analysis of these three analytes in Table 2. As evident from the results, the total analysis time and sensitivity are higher compared to these methods.[7,45–47] Moreover, this is the first UPLC-MS/MS method for the simultaneous determination of these three analytes in human plasma. Further, the plasma volume used for processing is also very low compared to existing methods. Karra et al.[24] have reported a sensitive method for the simultaneous determination of LOS, EXP-3174, and amlodipine in human plasma. Moreover, the method was used for the pharmacokinetic analysis of only LOS and EXP-3174 in six healthy subjects. In the present work, sensitivity achieved for LOS (0.5 ng/mL) was identical, while for EXP-3174 it was two times less compared to the previous work.[24] However, the volume of plasma required for processing was half of that used in this reported work.[24] Additionally, these two analytes were not baseline resolved and the analysis time was 2.5 min per sample,[24] whereas the chromatographic analysis time was 2.4 min in the present study.

Table 3. Intrabatch and interbatch precision and accuracy for losartan, EXP-3174, and hydrochlorothiazide.

QC level (nominal concentration, ng/mL)	Intrabatch (n = 6; single batch)			Interbatch (n = 30; 6 from each batch)		
	Mean conc. observed (ng/mL)	% CV	% Accuracy	Mean conc. found for 5 batches (ng/mL)	% CV	% Accuracy
Losartan						
LLOQ QC (0.500)	0.481	2.88	96.2	0.482	2.36	96.4
LQC-2 (1.500)	1.498	1.62	99.9	1.493	1.39	99.5
LQC-1 (100.0)	102.7	3.84	102.7	102.8	2.17	102.8
MQC-2 (200.0)	206.8	4.06	103.4	194.9	5.09	97.5
MQC-1 (300.0)	303.0	2.92	101.0	294.8	4.68	98.3
HQC (400.0)	379.8	3.76	95.0	404.6	3.75	101.2
EXP-3174						
LLOQ QC (1.000)	1.017	4.93	101.7	0.991	1.25	99.1
LQC-2 (3.000)	3.093	1.72	103.1	3.041	1.97	101.4
LQC-1 (150.0)	144.7	3.72	96.5	151.2	3.36	100.8
MQC-2 (300.0)	295.9	2.37	98.6	282.7	3.17	94.2
MQC-1 (450.0)	448.5	1.87	99.7	463.6	2.25	103.0
HQC (600.0)	612.0	3.11	102.0	586.4	1.54	97.7
Hydrochlorothiazide						
LLOQ QC (0.250)	0.247	1.43	98.8	0.255	1.56	102.0
LQC-2 (0.750)	0.759	2.11	101.2	0.768	3.18	102.4
LQC-1 (30.0)	29.2	3.18	97.3	29.6	4.05	98.7
MQC-2 (60.0)	61.8	4.47	103.0	59.6	2.26	99.3
MQC-1 (90.0)	89.4	3.15	99.3	86.9	4.71	96.6
HQC (120.0)	118.5	2.30	98.8	122.1	3.23	101.8

CV: Coefficient of variation; LLOQ: lower limit of quantitation; LQC: low quality control; MQC: medium quality control; HQC: high quality control.

2.2. Results for method validation

The autosampler carryover or memory effects can have consequential effects during chromatographic separation and can dramatically limit the dynamic range and precision of the assay. The results obtained in this study showed minimal carryover of analyte ($\leq$0.15% of LLOQ area) in the extracted blank sample after injection of upper limit of quantitation (ULOQ) sample for the analytes.

The method was highly selective for the determination of all the analytes. Representative MRM ion chromatograms in Figures 3 and 4 of (a) blank human plasma with IS, (b) at LLOQ, and IS (c) in real subject sample for LOS, EXP-3174, and HCTZ, respectively, demonstrate the selectivity of the method. The ion chromatograms showed good peak shape with no interference peak of endogenous components at the retention times of analytes and ISs. Moreover, there was no interference due to commonly used medications during quantitation of the analytes under the MRM mode.

The calibration curves were linear over the validated concentration range of 0.5–500, 1.0–750, and 0.25–150 ng/mL with the correlation coefficient value, $r^2 \geq 0.9989$, ≥ 0.9988, and ≥ 0.9979 for LOS, EXP-3174, and HCTZ, respectively. The equations for means (n = 5) of five calibration curves were $y = (0.003342 \pm 0.000047)$ x – (0.000055 ± 0.000077), $y = (0.002228 \pm 0.000051)$ x+ (0.000055 ± 0.000144), and $y = (0.011199 \pm 0.000163)$ x + (0.000194 ± 0.000286) for LOS, EXP-3174, and HCTZ, respectively. The accuracy and precision (% CV) for the calibration curve standards were 97.7%–102.7% and 0.95–5.45 for LOS, 98.3%–101.3% and 1.18–7.24 for EXP-3174, and 96.9%–104.5% and 1.28–5.59 for HCTZ, respectively. The LLOQ in the standard curve for the analytes was measured at a signal-to-noise ratio (S/N) of $\geq$22. The intrabatch and interbatch precision (% CV) across six quality control samples ranged from 1.25 to 5.09 over the analytical range and the accuracy was within 94.2% to 103.4% for all the analytes (Table 3).

Table 4. Extraction recovery of losartan, EXP-3174, and hydrochlorothiazide from human plasma.

QC level	Losartan			EXP-3174			Hydrochlorothiazide		
	Area response		Extraction	Area response		Extraction	Area response		Extraction
	A	B	recovery, % (B/A)	A	B	recovery, % (B/A)	A	B	recovery, % (B/A)
LQC-2	924	913	98.8	1193	1182	99.0	1379	1289	93.5
LQC-1	65,512	64,534	98.5	60,123	59,182	98.4	59,470	57,270	96.3
MQC-2	123,577	126,689	102.5	137,734	128,093	93.0	121,473	114,670	94.4
MQC-1	189,764	188,985	99.6	210,049	197,866	94.2	173,718	158,778	91.4
HQC	262,359	252,157	96.1	251,929	246,387	97.8	257,549	227,931	88.5

QC level	Candesartan (IS)			Hydroflumethiazide (IS)		
	Area response		Extraction	Area response		Extraction
	A	B	recovery, % (B/A)	A	B	recovery, % (B/A)
LQC-2	182,306	184,810	101.4	111,874	106,728	95.4
LQC-1	191,242	194,126	101.5	125,652	112,835	89.8
MQC-2	196,076	189,010	96.4	114,223	109,882	96.2
MQC-1	194,876	195,762	100.4	120,396	111,728	92.8
HQC	197,148	187,014	94.8	122,379	105,491	86.2

LQC: low quality control; MQC: medium quality control; HQC: high quality control;

A: Mean area response of six replicate samples prepared by extracting spiked blank plasma;

B: Mean area response of six replicate samples prepared by spiking in extracted blank plasma

The mean extraction recovery for all the analytes and ISs is presented in Table 4. The recovery was 96.1%–102.5% for LOS, 93.0%–99.0% for EXP-3174, and 88.5%–96.3% for HCTZ across five QC levels. The presence of unmonitored, co-eluting compounds from the matrix may result in ion suppression/enhancement, decrease/increase in sensitivity of analytes over a period of time, increased baseline, and drift in retention time and thus can compromise the overall reliability of a validated method. In the present work, no ion suppression effects were observed under the optimized sample preparation and chromatographic conditions. Results of the postcolumn analyte infusion experiment showed negligible ion suppression or enhancement at the retention time of analytes and ISs (Figure 5a–5e). Further, quantitative evaluation of the matrix effect was also carried out for all the analytes and ISs from the peak area response and expressed as matrix factors as shown in Table 5. The IS-normalized matrix factors (analyte/IS) were 0.92–0.98 for LOS, 0.91–1.06 for EXP-3174, and 0.93–1.09 for HCTZ. Further, the matrix effect was also checked in lipemic and hemolyzed plasma samples in addition to normal K_3EDTA plasma by calculating the precision (% CV) in the measurement of the slope of calibration curves. The % CV values of the slopes of calibration lines in ten different plasma lots were 2.32, 2.29, and 3.19 for LOS, EXP-3174, and HCTZ, respectively.

Table 5. Matrix factor for losartan, EXP-3174, and hydrochlorothiazide.

QC level	Losartan			EXP-3174			Hydrochlorothiazide		
	Area response		Matrix factor (B/A)	Area response		Matrix factor (B/A)	Area response		Matrix factor (B/A)
	A	B		A	B		A	B	
LQC-2	967	913	0.944	1256	1182	0.941	1372	1289	0.939
LQC-1	64,340	64,534	1.003	62,825	59,182	0.942	54,908	57,270	1.043
MQC-2	127,325	126,689	0.995	125,704	128,093	1.019	109,837	114,670	1.044
MQC-1	193,632	188,985	0.976	188,443	197,866	1.050	164,707	158,778	0.964
HQC	258,888	252,157	0.974	251,415	246,387	0.980	219,586	227,931	1.038
QC level	Candesartan						Hydroflumethiazide		
	Area response		Matrix factor (B/A)				Area response		Matrix factor (B/A)
	A	B					A	B	
LQC-2	180,478	184,810	1.024				111,757	106,728	0.955
LQC-1	188,106	194,126	1.032				118,275	112,835	0.954
MQC-2	187,138	189,010	1.010				107,727	109,882	1.020
MQC-1	197,739	195,762	0.990				108,896	111,728	1.026
HQC	182,809	187,014	1.023				110,461	105,491	0.955

LQC: low quality control; MQC: medium quality control; HQC: high quality control;
A: Mean area response of six replicate samples prepared in mobile phase (neat samples);
B: Mean area response of six replicate samples prepared by spiking in extracted blank plasma

The stability of all the analytes in plasma was established at appropriate temperatures and storage periods required for clinical analysis. The stock solutions kept for short-term and long-term stability of analytes and ISs were found stable at room temperature up to 7 h and for a minimum period of 7 days, respectively. The detailed results for bench top stability, wet extracts, dry extracts, processed sample, and freeze-thaw and long-term stability of the analytes are summarized in Table 6. The precision and accuracy results found for method ruggedness with different columns and analysts were within 3.25% to 6.01% and 93.5% to 102.4% for LOS, 2.53% to 4.31%, and 96.8% to 100.9% for EXP-3174 and 4.17% to 5.95% and 98.3% to 104.7% for HCTZ,

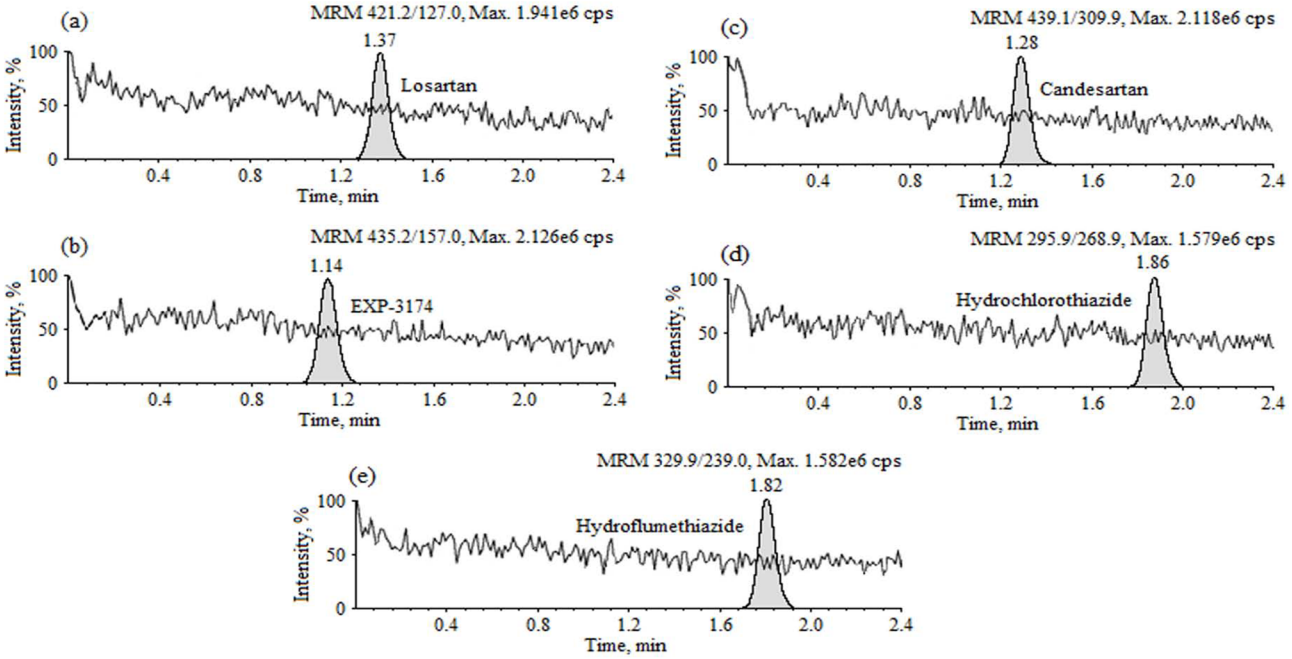

Figure 5. Postcolumn analyte infusion MRM LC-MS/MS chromatograms for (a) losartan, (b) EXP-3174, (c) candesartan, (d) hydrochlorothiazide, and (e) hydroflumethiazide.

respectively. The dilution reliability test was performed to validate analyte concentrations that are above the ULOQ of the linear range, especially with regard to clinical samples. The precision (% CV) and accuracy values for 1/10th dilution were 2.61–6.38 and 93.5–106.3 for all the analytes.

2.3. Application of the method in healthy subjects and incurred sample reanalysis

The proposed UPLC-MS/MS method was successfully applied for a comparative bioavailability study of fixed dose tablet formulation containing 50 mg of LOS and 12.5 mg of HCTZ in 65 healthy subject samples under fasting. The mean plasma concentration vs. time profiles of LOS, EXP-3174, and HCTZ after oral administration of the drugs are presented in Figure 6. The important pharmacokinetic parameters of the study like C_{max}, AUC_{0-48}, AUC_{0-inf}, T_{max}, K_{el}, and $t_{1/2}$ were calculated for LOS, EXP-3174, and HCTZ and the values for test and reference formulations are given in Table 7. The results obtained were in good agreement with reported studies in Indian[7] and Chinese subjects.[47] The ratios of mean log-transformed parameters and their 90% confidence intervals varied from 90.7% to 101.9% for LOS, 95.3% to 107.2% for EXP-3174, and 94.3% to 104.2% for HCTZ, which are within the acceptance range of 80%–125%. From these results the two formulations can be considered equivalent for both the rate and extent of drug absorption. Thus, the developed assay procedure for LOS, EXP-3174, and HCTZ in human plasma samples demonstrates the precision and sensitivity needed for pharmacokinetic studies of these drugs.

Incurred sample reanalysis (ISR) is conducted to assess the reproducibility of a validated method by reanalysis of selected subject samples after initial analysis is completed.[48] A well-established bioanalytical method with an integrated ISR plan can lead to continuous review of assay performance. The ISR test reinforces the confidence in a method if reproducibility is demonstrated in the study sample analysis and has now become mandatory for bioanalytical assays. The ISR results are expressed as percentage change, where

Table 6. Stability of losartan, EXP-3174, and hydrochlorothiazide in human plasma under different conditions.

Storage conditions	Losartan		EXP-3174		Hydrochlorothiazide	
	Mean of six stability samples (ng/mL) ± SD	% Change	Mean of six stability samples (ng/mL) ± SD	% Change	Mean of six stability samples (ng/mL) ± SD	% Change
Bench top stability at room temperature; 14 h						
LQC-2	1.516 ± 0.012	1.07	2.89 ± 0.176	–3.67	0.745 ± 0.022	–0.67
LQC-1	102.4 ± 5.68	2.40	147.3 ± 10.32	–1.80	30.5 ± 4.015	1.67
HQC	397.9 ± 15.9	–0.53	615.6 ± 12.87	2.60	118.8 ± 6.97	–1.00
Wet extract stability; 5 °C, 24 h						
LQC-2	1.474 ± 0.018	–1.73	3.13 ± 0.065	4.33	0.768 ± 0.016	2.40
LQC-1	106.7 ± 7.13	6.70	152.3 ± 9.67	1.53	28.6 ± 6.33	–4.67
HQC	408.0 ± 15.2	2.00	592.5 ± 14.32	–1.25	122.1 ± 7.69	1.75
Dry extract stability at 5 °C, 62 h						
LQC-2	1.521 ± 0.019	1.40	3.06 ± 0.069	2.00	0.758 ± 0.017	1.07
LQC-1	98.9 ± 6.25	–1.10	155.4 ± 8.75	3.60	32.0 ± 4.75	6.67
HQC	410.2 ± 14.3	2.55	608.9 ± 12.40	1.48	126.5 ± 5.16	5.42
Processed sample stability at 25 °C, 42 h						
LQC-2	1.472 ± 0.024	–1.87	3.05 ± 0.197	1.67	0.757 ± 0.018	0.93
LQC-1	104.2 ± 7.33	4.20	146.0 ± 7.62	–2.67	30.7 ± 6.31	2.33
HQC	413.5 ± 24.0	3.38	618.0 ± 11.94	3.00	114.6 ± 8.65	–4.50
Freeze & thaw stability in plasma; 6 cycles, –20 °C						
LQC-2	1.535 ± 0.019	2.33	2.97 ± 0.034	–1.00	0.727 ± 0.019	–3.07
LQC-1	105.4 ± 5.97	5.40	156.6 ± 9.64	4.40	29.6 ± 4.52	–1.33
HQC	386.9 ± 13.9	–3.28	606.3 ± 18.66	1.05	121.8 ± 5.35	1.50
Freeze & thaw stability in plasma; 6 cycles, –70 °C						
LQC-2	1.531 ± 0.018	2.07	3.11 ± 0.412	3.67	0.781 ± 0.011	4.13
LQC-1	98.9 ± 4.44	–1.10	158.7 ± 8.14	5.80	29.4 ± 3.47	–2.00
HQC	407.3 ± 11.3	1.83	594.2 ± 19.36	–0.97	121.0 ± 8.32	0.83
Long-term stability in plasma; 60 days, –20 °C						
LQC-2	1.481 ± 0.015	–1.27	3.18 ± 0.126	6.00	0.741 ± 0.014	–1.320
LQC-1	104.7 ± 6.49	4.70	148.9 ± 7.14	–0.73	30.5 ± 1.32	1.67
HQC	375.9 ± 15.6	–6.03	617.6 ± 16.26	2.93	116.7 ± 4.08	–2.75
Long-term stability in plasma; 60 days, –70 °C						
LQC-2	1.571 ± 0.021	4.73	2.88 ± 0.181	–4.00	0.773 ± 0.015	3.07
LQC-1	97.4 ± 6.43	–2.60	151.3 ± 7.74	0.87	28.3 ± 3.68	–5.67
HQC	406.8 ± 17.4	1.70	595.6 ± 12.68	–0.73	123.6 ± 4.89	3.00

LQC: low quality control; HQC: high quality control; SD: Standard deviation; n: Number of replicates at each level

$$\% \ \text{Change} = \frac{\text{Repeat value - Original value}}{\text{Mean of original and repeat values}} \times 100$$

In the present work, the assay reproducibility test conducted with 213 incurred samples showed % change within ±18% of the initial analysis results for all the analytes, which confirms the reproducibility of the proposed method.

Table 7. Mean pharmacokinetic parameters following oral administration of 50 mg losartan and 12.5 mg hydrochlorothiazide test and reference formulation to 65 healthy Indian subjects under fasting conditions.

Parameter	Mean ± SD		Ratio (test/ reference),%	90% CI (Lower-Upper)	Power	Intrasubject variation, % CV
	Test	Reference				
Losartan						
C_{max} (ng/mL)	243 ± 53	251 ± 69	96.9	93.3–101.9	0.9991	9.14
AUC $_{0-48}$ (h. ng/mL)	517 ± 194	552 ± 213	93.9	90.7–97.8	0.9996	8.27
AUC $_{0-inf}$ (h. ng/mL)	535 ± 209	573 ± 256	94.1	91.1–98.2	0.9995	9.63
T_{max} (h)	1.46 ± 0.29	1.52 ± 0.35	–	–	–	–
$t_{1/2}$ (h)	2.12 ± 1.05	2.37 ± 1.11	–	–	–	–
Kel (1/h)	0.322 ± 0.007	0.301 ± 0.006	–	–	–	–
EXP-3174						
C_{max} (ng/mL)	455 ± 152	463 ± 178	98.4	95.3–102.9	0.9998	5.44
AUC $_{0-48}$ (h. ng/mL)	2147 ± 784	2102 ± 693	101.9	97.7–104.8	0.9994	7.97
AUC $_{0-inf}$ (h. ng/mL)	2235 ± 739	2173 ± 556	102.6	98.1–107.2	0.9995	9.13
T_{max} (h)	3.28 ± 0.33	3.15 ± 0.42	–	–	–	–
$t_{1/2}$ (h)	4.05 ± 0.84	4.96 ± 0.95	–	–	–	–
Kel (1/h)	0.176 ± 0.021	0.140 ± 0.015	–	–	–	–
Hydrochlorothiazide						
C_{max} (ng/mL)	65.32 ± 23.4	66.41 ± 19.3	98.5	94.3–103.9	0.9998	4.14
AUC $_{0-48}$ (h. ng/mL)	417 ± 134	425 ± 153	98.1	96.7–103.8	0.9994	6.17
AUC $_{0-inf}$ (h. ng/mL)	455 ± 139	463 ± 156	97.9	95.1–104.2	0.9995	6.43
T_{max} (h)	2.40 ± 0.18	2.53 ± 0.22	–	–	–	–
$t_{1/2}$ (h)	9.12 ± 1.20	9.08 ± 1.25	–	–	–	–
Kel (1/h)	0.0760 ± 0.003	0.0763 ± 0.004	–	–	–	–

C_{max}: Maximum plasma concentration; AUC_{0-t}: Area under the plasma concentration-time curve from zero hour to 48 h;
AUC_{0-inf}: Area under the plasma concentration-time curve from zero hour to infinity; T_{max}: Time point of maximum plasma concentration;
$t_{1/2}$: Half life of drug elimination during the terminal phase; K_{el}: Elimination rate constant; SD: Standard deviation; CI: confidence interval;
CV: coefficient of variation

3. Experimental

3.1. Chemicals and materials

Reference standards of LOS potassium (99.6%) and HCTZ (99.3%) were obtained from United States Pharmacopeia (Rockville, MD, USA). EXP-3174 (98.6%), CAN (99.6%), and HFMZ (99.4%) were procured from Toronto Research Chemicals Inc. (Ontario, Canada). Suprapure grade *ortho*-phosphoric acid, ammonium formate, and formic acid were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). Oasis HLB (1 cc, 30 mg) extraction cartridges were from Waters Corporation (Milford, MA, USA). Water used in the study was prepared using the Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma in K_3EDTA was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20 °C until use.

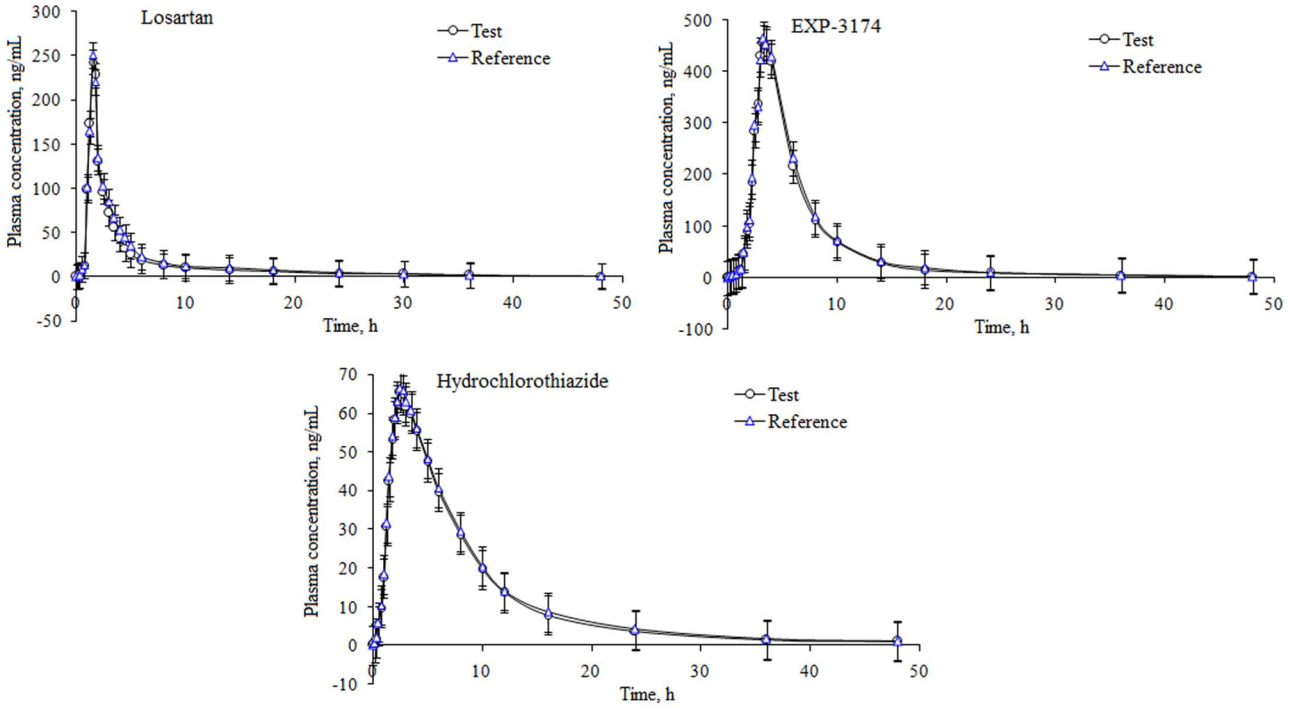

Figure 6. Mean plasma concentration-time profiles of losartan, EXP-3174, and hydrochlorothiazide after oral administration of 50 mg of losartan potassium and 12.5 mg of hydrochlorothiazide hydrochloride fixed dose tablet formulation to 65 healthy Indian male subjects under fasting.

3.2. Liquid chromatography and mass spectrometric conditions

The chromatographic analysis of LOS, EXP-3174, and HCTZ was carried out on a Waters Acquity UPLC system (Milford, MA, USA) employing a BEH C18 (50 × 2.1 mm, 1.7 μm) column, maintained at 35 °C. The mobile phase was prepared in premixed solvents consisting of 1.0 % (v/v) formic acid in water and acetonitrile (15:85, v/v). The mobile phase was pumped at a flow rate of 0.350 mL/min. The injection volume was set at 10 μL. The sample manager temperature was maintained at 5 °C with an alarm band of ±3 °C and the average pressure of the system was 6000 psi. Detection and quantitation of analytes and ISs was carried out using multiple reaction monitoring (MRM) for deprotonated precursor → product ion transitions on a Quattro Premier XE mass spectrometer from Waters – Micro Mass Technologies (Milford, MA, USA), in the negative ionization mode. Source dependent and compound dependent mass parameters optimized and MRM transitions for analytes and ISs are summarized in Table 1. MassLynx software version 4.1 was used to control all parameters of UPLC and MS.

3.3. Calibrators and quality control samples

Separate stock solutions (1.0 mg/mL) of LOS, EXP-3174, and HCTZ were prepared by dissolving their accurately weighed amounts in methanol. Their intermediate solutions of 100.0 μg/mL for LOS and EXP-3174 and 50.0 μg/mL for HCTZ were prepared in methanol:water (50:50, v/v). Calibration standards (CSs) and quality control (QC) samples were made by spiking blank plasma with suitable volumes of working solutions prepared from intermediate solutions for all the analytes. The mixed CSs in plasma were prepared at 0.50, 1.0, 2.5, 5.0, 10.0, 25.0, 75.0, 150, 300, and 500 ng/mL for LOS; 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100, 250, 500, and 750 ng/mL

for EXP-3174; and 0.25, 0.50, 1.0, 2.0, 5.0, 15.0, 30.0, 50.0, 100, and 150 ng/mL for HCTZ. QC samples were prepared at 400.0/600.0/120.0 ng/mL (HQC, high quality control), 300.0/450.0/90.0 ng/mL (MQC-1, medium quality control-1), 200.0/300.0/60 ng/mL (MQC-2, medium quality control-2), 100.0/150.0/30.0 ng/mL (LQC-1, low quality control-1), 1.5/3.0/0.750 ng/mL (LQC-2, low quality control-2), and 0.50/1.00/0.25 ng/mL (LLOQ QC, lower limit of quantitation quality control) for LOS/EXP-3174/HCTZ, respectively. The stock solutions of ISs (1.0 mg/mL) were prepared by dissolving requisite amounts in methanol. Their combined working solutions (500 ng/mL for CAN and 50 ng/mL for HFMZ) were prepared by appropriate dilution of their stock solutions in methanol:water (50:50, v/v). All standard stock and working solutions used for spiking were stored at 5 °C, while CSs and QC samples in plasma were kept at –70 °C until use.

3.4. Sample extraction protocol

Prior to analysis, all calibration and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 μL of spiked plasma sample/subject sample, 50 μL of internal standard was added and vortexed for approximately 10 s. Further, 500 μL of 5% (v/v) *ortho*-phosphoric acid was added and vortexed for another 10 s. The samples were then loaded on Oasis HLB extraction cartridges that were preconditioned with 1.0 mL of methanol followed by 1.0 mL of water. Thereafter the cartridges were washed with 1.0 mL of 5% methanol in water and then with 1.0 mL of 5 mM ammonium formate followed by drying for 2 min by applying 1.72×10^5 Pa positive pressure of nitrogen at 2.4 L/min flow rate. Elution of analytes and ISs from the cartridges was carried out with 500 μL of acetonitrile:water (90:10, v/v) into prelabeled tubes. The eluate was evaporated to dryness in a thermostatically controlled water bath maintained at 40 °C under a gentle stream of nitrogen for 5 min. After drying, the residue was reconstituted in 100 μL of reconstitution solution [10 mM ammonium formate: acetonitrile (20:80, v/v)] and 10 μL was used for injection in the chromatographic system.

3.5. Validation procedures

Method validation procedures were based on USFDA guidelines[49] and are similar to those in our previous work.[50] A system suitability test was done to authenticate optimum instrument performance (e.g., sensitivity and chromatographic retention) and was performed by analyzing a reference standard solution prior to running the analytical batch. In this test, six consecutive injections of aqueous standard mixture of analytes (at ULOQ) and IS were injected at the start of each batch during method validation. The precision (% CV) in the measurement of area response and retention time was assessed. The precision (% CV) of the system suitability test was observed in the range of 0.13% to 0.24% for the retention time and 0.85% to 2.96% for the area response for all the analytes and ISs. Additionally, the accuracy in the measurement of solution concentration was also evaluated. System performance was checked by calculating the signal to noise ratio for quantifying LLOQ sample for all the analytes. In this experiment, one extracted blank (without analytes and IS) and one processed LLOQ sample with IS was injected at the beginning of each analytical batch. The signal to noise ratio for system performance was $\geq$22 for all the analytes. Autosampler carryover was evaluated by sequentially injecting aqueous standard of analytes, mobile phase, extracted blank plasma, ULOQ sample, two extracted blank plasma samples, LLOQ sample, and extracted blank plasma at the start of each batch.

Selectivity of the method towards endogenous plasma matrix components was verified in ten batches (8 normal lots of K_3EDTA, 1 hemolyzed, and 1 lipemic) of blank human plasma. The area response of analytes and ISs at their respective retention times was compared with the area response observed in the LLOQ samples,

prepared from the same lot of blank plasma. In addition, interference due to some commonly used medications like paracetamol, chlorpheniramine maleate, diclofenac, caffeine, acetylsalicylic acid, and ibuprofen by human volunteers was also checked. Their stock solutions (100 μg/mL) were prepared in methanol. Further, their working solutions (100 ng/mL) were prepared in methanol:water (50:50, v/v) and 10 μL was injected to check for any possible interference at the retention time of analytes and ISs.

The linearity of the method was determined by analysis of five linearity curves containing ten nonzero concentrations in the concentration range of 0.5–500 ng/mL for LOS, 1.0–750 ng/mL for EXP-3174, and 0.25–150 ng/mL for HCTZ. The area ratio response for analyte/IS obtained from multiple reaction monitoring was used for regression analysis. The simple linear equation $y = mx + c$ was used for regression analysis of spiked plasma calibration standards with reciprocal of the drug concentration (1/concentration) as a weighing factor $(1/x^2)$. The lowest standard on the calibration curve was accepted as the LLOQ if the analyte response was at least ten times more than that of extracted blank plasma.[49]

Intrabatch accuracy and precision were determined by analyzing six replicates of QC samples along with calibration curve standards on the same day. The interbatch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive days. Sample injection reproducibility was also checked by reinjecting one entire validation batch. Reinjection reproducibility for extracted samples was also checked by reinjection of an entire analytical run after storage at 5 °C.

Ion suppression/enhancement effects on MRM LC-MS/MS sensitivity were evaluated by postcolumn analyte infusion technique. A standard solution containing LOR, EXP-3174, HCTZ (at MQC-1 level), and ISs was infused postcolumn into the mobile phase at 10 μL/min. Aliquots of 10 μL of extracted control blank plasma sample were then injected into the column and chromatograms were acquired for the analytes. Extraction recovery of the analytes and ISs from human plasma was evaluated in six replicates by comparing the mean peak area responses of preextraction fortified samples to those of postextraction fortified samples representing 100% recovery. The matrix effect, expressed as matrix factors (MFs), was assessed by comparing the mean area response of postextraction fortified samples with the mean area of solutions prepared in mobile phase solutions (neat standards). IS-normalized MFs (analyte/IS) were calculated to assess the variability of the assay due to matrix effects. Relative matrix effect was assessed from the precision (% CV) values of the slopes of the calibration curves prepared from ten different plasma lots/sources. To prove the absence of the matrix effect, % CV should be less than 3%–4% as recommended for method applicability to support clinical studies.[51]

The standard stock solutions of analytes and ISs were evaluated for short-term and long-term stability at 25 °C and 5 °C, respectively. The analyte stability in spiked plasma samples was evaluated by measuring the area ratio response (analyte/IS) of stability samples against freshly prepared standards having identical concentration. The % change was determined using the expression

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

Bench top (at room temperature), wet extract at 5 °C, dry extract at 5 °C, processed sample stability at room temperature, and freeze-thaw (–20 °C and –70 °C) and long-term (–20 °C and –70 °C) stability of analytes in plasma were studied at three QC levels using six replicates. The stability samples were quantified against freshly prepared quality control samples. Stability data were acceptable if the % CV of the replicate determinations did not exceed 15.0% and the mean accuracy value was within ±15.0% of the nominal value.

A ruggedness study is usually performed to facilitate transfer of a method to another laboratory; however, it can also be carried out during method development to assess the method's inherent variability. It provides a more rigorous approach for assessing method precision than a comparative intermediate precision study normally performed as part of method validation. In the present work, method ruggedness was evaluated to check any possible variation from analyst to analyst and column to column (two different columns of the same make having different batch numbers) using the same samples (two precision and accuracy batches), while keeping the optimized method parameters constant. The degree of reproducibility was evaluated and expressed in terms of precision (% CV). The ability to dilute samples that have concentrations above the upper limit of the calibration range was validated by analyzing six replicate samples containing 1000/1500/300 ng/mL LOS/EXP-3174/HCTZ after ten-fold dilution, respectively. The precision and accuracy for dilution reliability were determined by comparing the samples against freshly prepared calibration curve standards.

3.6. Bioequivalence study and incurred sample reanalysis

A bioequivalence study was performed in 65 healthy subjects with fixed dose 50 mg LOS potassium + 12.5 mg HCTZ hydrochloride test (Generic Company, India) and reference (HYZAAR, Merck Sharp & Dohme, NJ, USA) tablet formulation. The primary target variables of the study were C_{max}, AUC_{0-48}, and AUC_{0-inf}, which were analyzed using the confidence interval approach. 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 performed as per the guidelines of the International Conference on Harmonization and USFDA.[52] The health checkup for all subjects was done by general physical examination, ECG, and laboratory tests like hematology, biochemistry, and urine examination. All subjects were negative for HIV, HBsAg, and HCV. They were orally administered 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 K_3EDTA as anticoagulant at predose (0.0 h), 0.17, 0.33, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0, 10.0, 12.0, 16.0, 24.0, 30.0, 36.0, and 48.0 h of administration of drug. Blood samples were centrifuged at 3200 rpm for 10 min and plasma was separated and stored at –70 °C until use. The pharmacokinetic parameters of LOS, EXP-3174, and HCTZ were estimated by noncompartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA). To determine whether the test and reference formulations were pharmacokinetically equivalent, C_{max}, AUC_{0-48}, and AUC_{0-inf} and their ratios (test/reference) using log transformed data were assessed. The drug formulations were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically nonsignificant ($P \geq 0.05$) and the 90% confidence intervals (CIs) for these parameters were within 80% to 125%.

The method reproducibility was verified by reanalysis of 213 incurred samples and the results were compared with original study samples. The acceptance criterion was based on two-thirds of the original results and repeat results should be within 20% of each other.[48]

3.7. Conclusion

As compared with earlier LC-MS/MS methods for the determination of these drugs, the proposed UPLC-MS/MS offers several advantages such as higher sensitivity, short analysis time, small plasma volume for processing, and low consumption of toxic organic solvents. The extraction method was suitably optimized for quantitative and reproducible recovery of the analytes having different physico-chemical properties. The matrix effect was extensively studied through postcolumn infusion, postextraction spiking, and calculation of slope of calibration

lines for the relative matrix effect. The method showed acceptable accuracy and precision in the measurement of these drugs in clinical samples. Finally, the method reproducibility was successfully demonstrated by reanalysis of incurred study samples, which has not been reported in previous methods.

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