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Liquid chromatography–mass spectrometric determination of losartan and its active metabolite on dried blood spots

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ABSTRACT

A simple and rapid quantitative bioanalytical liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for simultaneous determination of losartan and its active metabolite, losartan carboxylic acid on rat dried blood spots was developed and validated as per regulatory guidelines. Losartan and its metabolite were extracted from dried blood spots using 50% aqueous methanol and separated on Waters XTerra® RP18 (250 mm × 4.6 mm, 5 μm) column using mobile phase composed of 40% acetonitrile and 60% aqueous ammonium acetate (10 mM). The eluents were monitored using ESI tandem mass spectrometric detection with negative polarity in MRM mode using ion transitions *m/z* 421.2 → 179.0, *m/z* 435.3 → 157.0 and *m/z* 427.3 → 193.0 for losartan, losartan carboxylic acid and Irbesartan (internal standard), respectively. The method was validated over the linear range of 1–200 ng/mL and 5–1000 ng/mL with lower limits of quantification of 1.0 ng/mL and 5.0 ng/mL for losartan and losartan carboxylic acid, respectively. Inter and intra-day precision and accuracy (Bias) were below 5.96% and between −2.8 and 1.5%, respectively. The mean recoveries of the analytes from dried blood spots were between 89% and 97%. No significant carry over and matrix effects were observed. The stability of stock solution, whole blood, dried blood spot and processed samples were tested under different conditions and the results were found to be well within the acceptable limits. Additional validation parameters such as influence of hematocrit and spot volume were also evaluated and found to be well within the acceptable limits.

1. Introduction

Hypertension is one of the most important risk factors for cardiovascular disorders and has become an increasingly important contributor to the global health burden [1,2]. It is one of the most important causes of mortality and morbidity in the modern world [3]. Arrays of cardiovascular drugs are available to treat this variety of disorders by targeting the heart and blood vessels in multiple ways. Among the anti-hypertensives that lower blood pressure, losartan (LOS) 2-n-butyl-4-chloro-1-[p-(o-1H-tetrazol-5-ylphenyl)benzyl]-imidazole-5-metha-nol is a highly selective, orally active, non-peptide angiotensin II receptor antagonist indicated for the treatment of hypertension. It has a more potent active carboxylic acid metabolite (LCA) 2-n-butyl-4-chloro-1-[p-(o-1H-tetrazol-5-yl)biphenyl-4-l)methyl]imidazole-5-carboxyl acid [4].

Guthrie et al., first used dried blood spot (DBS) technique for detection of phenylketonuria in newborns [5]. It offers a number of advantages such as simpler sample collection, less invasive, requires a smaller blood volume, easier transport and storage over whole blood, plasma or serum collection techniques [6,7]. DBS has been used for screening of some inherited metabolic disorders in newborns [8–28] and it has emerged as an important technique for quantitative analysis of small molecules and their metabolites [29–37]. It has potential applications in many other fields like preclinical [38], pharmacokinetics [39–44], toxicokinetics [45] and extended to therapeutic drug monitoring of different classes of pharmaceutical active ingredients [46–52].

Several analytical methods were reported in the literature for analysis of LOS and LCA in plasma [53–61]; which include HPLC with UV [53], fluorescence [54,55] and mass spectrometry [56–61] as detectors. Sample treatment is usually carried out by solid phase extraction (SPE) [4,56–61]. To the best of authors' knowledge no work has been published for analysis of LOS and its active metabolite on DBS. Thus, the present study is the first report for quantification of LOS and its active metabolite LCA on DBS by LC–MS/MS.

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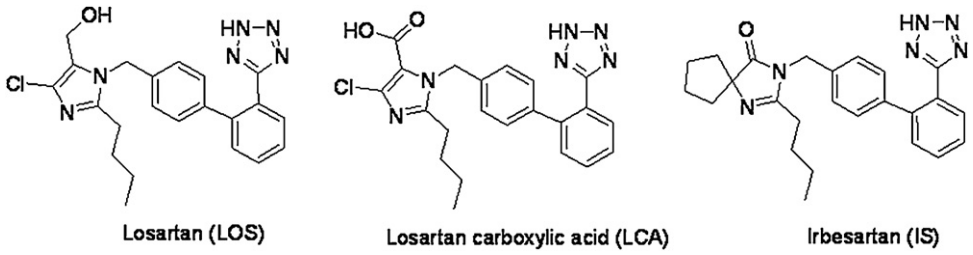

Fig. 1. Chemical structures of losartan, losartan carboxylic acid and Irbesartan (IS).

2. Experimental

2.1. Chemicals and materials

LOS, LCA and Irbesartan (IS) were purchased from M/s Varda Biotech, Mumbai, India. The chemical structures of LOS, LCA and IS are shown in Fig. 1. LC–MS grade methanol and acetonitrile were purchased from Merck India Pvt Ltd., Hyderabad, India. Control matrix (Na_2EDTA rat whole blood) was obtained from animal house, AU College of Pharmacy, Andhra University, Vishakapatnam, India. Ultrapure water was obtained from Milli-Q water purification system (Millipore Corp., Bedford, MA, USA) and solvents were filtered through a Millipore membrane filter (type HA, pore size 0.45 μm, Billerica, MA, USA). Before injection, all samples were passed through a disposable syringe filter (PTFE membrane filters, pore size 0.45 μm, Advantec MFS, Tokyo, Japan). Ammonium acetate supplied by Qualigens, Mumbai, India was used. Whattmann S&S 903 FTA blood spot cards were supplied by Whatmann (Sanford, USA). Sample tubes were obtained from Tarsons Pvt Ltd. (Kolkata, India).

2.2. Chromatographic conditions

The chromatography was carried out using an Agilent HPLC system (1100 series, Waldbronn, Germany), consisting G1312A Binary Pump, G1379A Dessager, G1329A Autosampler and G1329B Thermostat. The separation of analytes and IS was performed on Waters XTerra® RP18 (250 mm × 4.6 mm, 5 μm) using a mobile phase consisting of acetonitrile/10 mM ammonium acetate (40:60, v/v), pumped at a flow rate of 0.9 mL/min. The injection volume was 20 μL and the total analysis time per sample was 8.0 min.

2.3. Mass spectrometric conditions

Ionization and detection of analyte and IS was carried out on Agilent LC–MSD Trap SL mass spectrometer, Agilent (Waldbronn, Germany), equipped with an electrospray ion interface, operating in negative ion polarity. Quantification was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions m/z 421.2 → 179.0, m/z 435.3 → 157.0 and m/z 427.3 → 193.0 for LOS, LCA and IS, respectively (Fig. 2). Nitrogen was used as a nebulizer as well as curtain gas. Collision induced dissociation was achieved using helium gas. The ion source conditions were set as follows: temperature, 340 °C; nebulizer gas, 35 psi; dry gas, 9.0 L/min; ion spray voltage, 4500 V; collision energy, 35 V; electron multiplier voltage, 2300 V; declusturing potential, 60 V; focusing potential, 400 V; entrance potential, 10 V; collision exit potential, 30 V. All quantification data was processed using Quantanalysis version 1.5 software.

2.4. Preparation of stock, calibration standards and quality control (QC) solutions

The stock solutions of LOS, LCA were prepared by dissolving pure drug in methanol to yield concentrations of 1.0 mg/mL and 5.0 mg/mL, respectively. Similarly stock solution of IS were prepared by dissolving pure drug in methanol to yield concentration of 1.0 mg/mL. Further dilution of IS stock solution with methanol yielded IS working standard solution concentration of 10 μg/mL. The stock solutions were diluted with methanol to yield working standards of 10 μg/mL and 50 μg/mL of LOS and LCA, respectively. Calibration standards were prepared by diluting working standard solutions in 200 μL blank whole rat blood to yield 1, 2, 5, 10, 20, 50, 100, 200 ng/mL and 5, 10, 25, 50, 100, 250, 500, 1000 ng/mL of LOS and LCA, respectively. Quality control samples (LQC, MQC and HQC) were prepared in a similar manner to yield 4, 25, 150 ng/mL and 20, 125, 750 ng/mL of LOS and LCA, respectively. The samples were then mixed using a vortex mixer for 1 min.

2.5. Sample preparation

A 10 μL aliquot of each calibration standard and QC sample was spotted onto the circled area of FTA cards. The samples were allowed to dry at room temperature in the dark for at least 2 h. These calibration standards and QC samples were used in method validation. Using a puncher, a 3-mm single punch was manually made for each calibration standards, QC's and blank. The obtained disc was placed into clean tubes. The internal standard working solution (25 μL of 400 ng/mL) was added to all tubes except the blank to which a 25 μL aliquot of 50% aqueous methanol was added. 475 μL of 50% aqueous methanol was added to all the tubes and sonicated for 10 min followed by vortex for 5 min. The solutions were filtered through PTFE syringe filter with 0.45 μm. A 20 μL of these solutions was injected onto the LC–MS/MS system.

2.6. Bioanalytical method validation procedures

The method was validated according to EMEA guidelines [62].

2.6.1. Selectivity

The selectivity of the developed method was tested by analyzing six different batches of rat blank DBS samples. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectrometric conditions. The results were compared with those obtained with neat solution of the analyte at a concentration near the lower limit of quantification (LLOQ). This was to ensure that no interfering peaks were present in the biological matrix at the retention times of LOS and LCA.

2.6.2. Calibration curve

A volume of 10 μL of calibration standard solutions at 8 different concentrations were spiked on FTA cards and were subjected to the

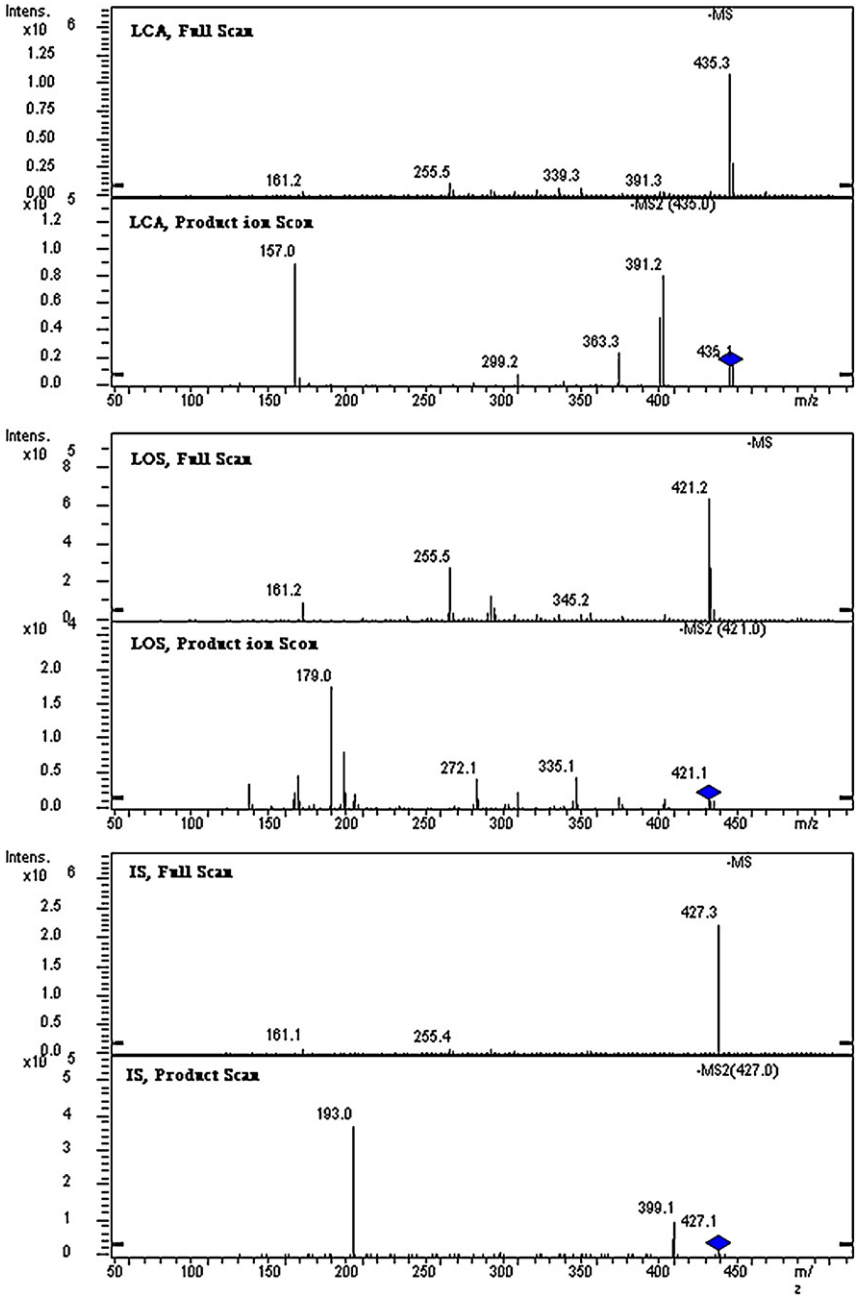

Fig. 2. Negative ion ESI-MS and MS/MS spectra of LCA, LOS and IS.

earlier described pre-treatment procedures and 25 μL of IS (working solution) was added to extracted sample. Finally 20 μL sample was injected into the LC–MS/MS. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios obtained were plotted against the corresponding concentrations of the analytes and the calibration curves set up by means of the least-square method.

2.6.3. Lower limit of quantification

The LLOQ is the lowest amount of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. The LLOQ was determined by spiking six samples with LOS and LCA at the lower end of the calibration curves (1.0 and 5.0 ng/mL, respectively). LLOQ was calculated from the regression equation of calibration curve.

2.6.4. Precision and accuracy

Quality control DBS samples containing low, medium and high concentrations of analyte were used to evaluate the precision and accuracy of the assay method. The intra-day assay precision and accuracy were obtained by analyzing six replicates of the QC samples in duplicate using a calibration curve constructed on the same day. The inter-day assay precision and accuracy were obtained by analyzing six quality control samples in duplicate using calibration curves constructed on 3 different days. The assay precision and accuracy was reflected by the percent coefficient of variation (% CV) and % bias, respectively.

2.6.5. Recovery

To determine the assay recovery, a 3-mm disc for three replicates of each DBS QC sample at concentrations of 4, 25 and 150 ng/mL of LOS and 20, 125 and 750 ng/mL of LCA were extracted

with 50% aqueous methanol using proposed extraction procedure. The results obtained for the extracted samples were compared to those of neat solutions (Aliquot 10 μL of each 4, 25, 150 ng/mL of LOS and 20, 125, 750 ng/mL of LCA was spiked in 490 μL of 50% aqueous methanol). Recovery was calculated using the following equation: % recovery = peak area of dried blood spot extract/peak area of neat solution × 100. Recovery was expressed in terms of % absolute recovery.

2.6.6. Stability

To test the stability of the analytes on DBS samples containing LOS and LCA at LQC and HQC were prepared from fresh rat whole blood as described in the DBS sample preparation section and stored at room temperature (24–26 °C). Stability of analytes on DBS cards were carried out over 30 days at room temperature. Stability experiments were performed for stock solution, whole blood at room temperature (24–26 °C) for 24 h. Stability of all analytes in processed samples (autosampler stability) was also investigated at 0 and 24 h at autosampler (4 °C). Each measurement was performed in triplicate.

2.6.7. Carry-over

Carryover caused by the residual analyte from injection of the previous sample was evaluated by injecting a reconstitution solvent blank immediately after the upper limit of quantitation (ULOQ) of the calibration curve. For sufficient accuracy at the LLOQ, any carryover peak must be less than 20% of the LLOQ response.

2.6.8. Dilution integrity

Dilution of the samples should not interfere with the accuracy and precision. To demonstrate the method is suitable for the analysis of DBS samples with analyte concentrations exceeding the ULOQ, the dilution integrity was assessed by diluting an extracted dilution DBS QC sample with extracted zero samples (containing IS only), followed by analysis with the calibration standards and regular QCs. The obtained bias (%) from the three replicates of the DBS QCs should be within ±15% of the nominal value.

2.6.9. Matrix effect

Neat solution of analytes and IS were spiked into three replicates of six different lots of extracted blank DBS samples at two concentration levels 4, 150 ng/mL and 20, 750 ng/mL for LOS and LCA, respectively. Peak areas of the above solutions were compared with those of respective neat solutions (Aliquot 10 of each 4, 150 ng/mL of LOS and 20, 750 ng/mL of LCA was spiked in 490 μL of 50% aqueous methanol). Matrix factor (MF) was calculated for each lot of matrix from the ratio of peak areas in the absence of matrix and the neat solution of analyte. IS-normalized MF was calculated by dividing MF of the analyte by MF of IS. The CV of IS-normalized MF should be less than 15%.

2.6.10. Spot volume

Aliquots of 10, 15, 20 μL of rat whole blood at LQC and HQC of both analytes were spotted onto FTA cards. The dried spots were extracted and analyzed. Concentrations of LOS and LCA were determined from calibration curves derived from 10 μL DBS.

2.6.11. Hematocrit value

Hematocrit (Hct) has a considerable effect on blood viscosity, and it may affect flux and diffusion properties of the blood put on filter paper used. In addition there may be a significant difference of analyte concentration between central and peripheral areas within the dried blood spot, due to chromatographic effects. Hematocrit is normally about 0.31–0.50 for rats [63]. At a high Hct value, the distribution of blood sample through the paper/card might be poor, resulting in a smaller blood spot when compared with the blood sample with a low Hct. The effect of hematocrit values 20%, 35% and 50% were tested for LOS and LCA at MQC.

3. Results and discussion

3.1. Method development

The chromatographic conditions were aimed to achieve an efficient separation and resolution of LOS, LCA and IS from endogenous peaks. Also, the response should be adequate with sharp peak shape and short run time, which includes selection of column, mobile phase and flow rate. During the optimization of chromatographic conditions, different ratios (v/v) of water/methanol and water/acetonitrile were tried as mobile phase, including formic acid, ammonium formate and ammonium acetate buffers in varying strengths on Waters Symmetry C_{18} (250 mm × 4.6 mm, 5 μm), Waters Atlantis dC_{18} (150 mm × 4.6 mm, 5 μm), Agilent Zorbax SBC_{18} (250 mm × 4.6 mm, 5 μm), Agilent Zorbax XDB C_{18} (250 mm × 4.6 mm, 5 μm) and Waters XTerra RP 18 (250 mm × 4.6 mm, 5 μm). In addition, the effect of flow rate was also studied for 0.6–1.2 mL/min, which was also responsible for acceptable chromatographic peak shapes. The use of Waters XTerra® RP 18 (250 mm × 4.6 mm, 5 μm) column helped in separation and elution of LOS, LCA and IS in a short time. The selection of mobile phase for their separation was very critical as they have similar retention behavior and retention time. The mobile phase consisting of 10 mM ammonium acetate solution:acetonitrile (60:40, % v/v) was found to be most appropriate for faster elution, improved efficiency and peak shape. The retention times for LCA, LOS and IS were 3.1, 4.5 and 5.3 min, respectively at a flow rate of 0.9 mL/min. The proposed method was aimed for preclinical studies which involve blood samples from animals not treated with Irbesartan previously. Thus the interference of Ibresartan from the study samples in preclinical studies was eliminated. However, it may be noted that the proposed assay can be used only for preclinical but not clinical studies.

The mass spectrometer was tuned to both positive and negative ionization modes with ESI for optimum response of LOS, LCA and IS. It was found that the intensity of negative ion was higher than that of the positive ion. In the full-scan spectra, the most abundant deprotonated ions $[M-H]^-$ were at m/z 421.2, 435.3 and 427.3 for LOS, LCA and IS, respectively. Parameters such as dry gas temperature, nebulizer gas pressure, dry gas flow, ion spray voltage, collision energy, declusturing potential voltage, entrance potential voltage and capillary voltage were optimized to obtain the highest intensity of product ion. The full scan and product ion scan spectra (Fig. 2) showed high abundance fragment ions at m/z 179.0, 157.0 and 193.0 for LOS, LCA and IS, respectively. Collision-induced dissociation (CID) was achieved using helium gas. The collision gas pressure and collision energy of collision-induced decomposition were optimized for maximum response of the fragmentation. The precursor → product ion transitions of m/z 421.2 → 179.0 for LOS, m/z 435.3 → 157.0 for LCA and m/z 427.3 → 193.0 for IS were chosen for MRM.

3.2. Sample pretreatment

Unlike plasma, whole blood is a complex heterogeneous matrix where the analytes are distributed between plasma and blood cells according to partition coefficients. Selection of a suitable solvent for extraction of LOS and LCA from DBS was carried out using various solvents like acetonitrile, methanol and different buffers. The organic solvents alone did not allow a good extraction of the analytes. Addition of aqueous solvent (water) increases extraction of the analytes from DBS samples. 50% aqueous methanol was finally

Table 1
Precision and accuracy data.

Analyte	concentration (ng/mL)	Intra-day			Inter-day		
		Mean ± S.D.	% CV	% bias	Mean ± S.D.	% CV	% bias
LOS	4	3.94 ± 0.18	4.66	−1.5	3.96 ± 0.22	5.51	−1.0
	25	24.60 ± 1.17	4.75	−1.6	24.30 ± 1.23	5.04	−2.8
	150	148.00 ± 3.87	2.61	−1.3	149.00 ± 3.93	2.64	−0.7
LCA	20	20.30 ± 1.12	5.52	1.5	19.60 ± 1.17	5.95	−2.0
	125	124.00 ± 2.96	2.39	−0.8	124.00 ± 3.13	2.52	−0.8
	750	749.00 ± 7.69	1.03	−0.1	748.00 ± 7.22	2.95	−0.3

chosen as the extraction solvent which enhanced the absolute recovery of all the analytes. Hence, aqueous methanol was used as extraction solvent which was simple, cost effective and without comprising the sensitivity of the method. Whereas, in other cases the extraction procedures SPE and LLE (t-methyl butyl ether and hexane) were used.

3.3. Validation

3.3.1. Selectivity

LOS and LCA were well separated from interferences in the matrix blank using the proposed LC–MS/MS method. No interference was observed in either drug-free DBS (matrix blank) or drug-free DBS spiked with the internal standard (zero sample).

3.3.2. Linearity and limit of quantification

The calibration curves (peak area ratio of analyte versus concentration) of LOS and LCA were found to be linear over the selected calibration range. The calibration data was subjected to least square regression analysis and the mean linear regression equation obtained for the proposed method was $y = 0.1538x + 0.336$ and $y = 0.0226x + 0.271$ for LOS and LCA, respectively. The correlation coefficients for LOS and LCA were highly significant with $r^2 = 0.9962$ and 0.9973, respectively. The method was validated over a linear range of 1–200 ng/mL and 5–1000 ng/mL for LOS and LCA, respectively, this linearity range was within the validation range of preclinical assay [22]. Lower Limit of quantification (LLOQ) for the validated method was 1.0 ng/mL and 5.0 ng/mL for LOS and LCA, respectively as % CV of accuracy and precision values of both the analytes were <15%. Typical MRM mass chromatograms of blank, Internal standard, LCA, 5 ng/mL and LOS, 1 ng/mL are shown in Fig. 3.

3.3.3. Precision and accuracy

The intra- and inter-day performance of the assay method was evaluated by analyzing six replicates each of DBS QC samples at 3 concentration levels of LQC, MQC and HQC on the 3 separate validation days. As shown in Table 1, the obtained precision (% CV) ranged from 2.6 to 4.7% for LOS, 1.0 to 5.5% for LCA and 2.6 to 5.5% for LOS, 1.0 to 5.9% for LCA, respectively, for the intra-day and inter-day evaluations. The accuracy ranged from −1.6 to −1.3% for LOS, −0.8 to 1.5% for LCA and −2.8 to −0.7% for LOS, −2.0 to −0.03% for LCA bias, for intra-day and inter-day batches, respectively.

3.3.4. Recovery and matrix effect

The absolute recoveries for both analytes of DBS samples at LQC, MQC and HQC were determined and found to be in the range of 89.13–97.97%. These results demonstrate (replicates $n = 3$) that the loss of sample during sample preparation was negligible. The CV of IS-normalized MF calculated from the six lots of matrix was less than 6%. Significant change in the intensity of the analyte signal was not observed due to matrix at the regular DBS QC sample concentrations tested. The matrix effect and recovery data are presented in Tables 2 and 3, respectively.

Table 2
Matrix effects on DBS samples.

Analyte	Concentration (ng/mL)	IS-normalized matrix effect	
		Mean ± S.D.	% CV
LOS	4	0.92 ± 0.05	5.43
	150	0.99 ± 0.04	4.04
LCA	20	1.04 ± 0.05	4.80
	750	1.02 ± 0.06	5.80

3.3.5. Stability

All stability tests showed sufficient stability of both analytes, LOS and LCA under various test conditions. Stability on FTA cards of both analytes showed no significant sample loss over 30 days at room temperature. Stability data for 10, 20 and 30 days were less than 15% bias. Stock solution and whole blood stability was tested at room temperature for 24 h. Moreover processed samples stability was done by re-injecting after 24 h storage in the autosampler at 4 °C and was stable up to 24 h. The results are given in Table 4, which indicate that significant sample loss was not shown under any of the test conditions.

3.3.6. Carry-over

Carry-over evaluation was performed to ensure that it does not affect the accuracy and precision of the proposed method. Almost negligible area (less than 5% of LLOQ area) was observed in blank plasma run after ULOQ, which suggests no carry-over of the analyte in subsequent runs. Moreover, other peaks not appeared during the analysis of blank samples.

3.3.7. Dilution integrity

Dilution integrity was assessed by 4-fold dilution of the DBS (800 ng/mL LOS and 4000 ng/mL LCA) QC sample were extracted and analyzed in three replicates along with calibration standards and regular QCs. The obtained bias (%) and CV (%) were −2.74% and 5.96%, respectively.

3.3.8. Spot volume

The assay accuracy and precision for LOS and LCA are given in Table 5, and found to be acceptable with less than 15%. The difference between the accuracy values derived from 15, 20 μL spots compared to those from 10 μL spot were less than 8.25% which

Table 3
Recovery data on DBS samples.

Analyte	Concentration (ng/mL)	*a*	*b*	Absolute recovery (%)
LOS	4	4.12	3.81	92.48
	25	24.84	22.14	89.13
	150	149.62	140.49	93.90
LCA	20	19.67	18.25	92.78
	125	124.16	121.64	97.97
	750	748.25	719.29	96.13

a: values obtained from neat solutions.
b: values obtained from DBS QC samples.

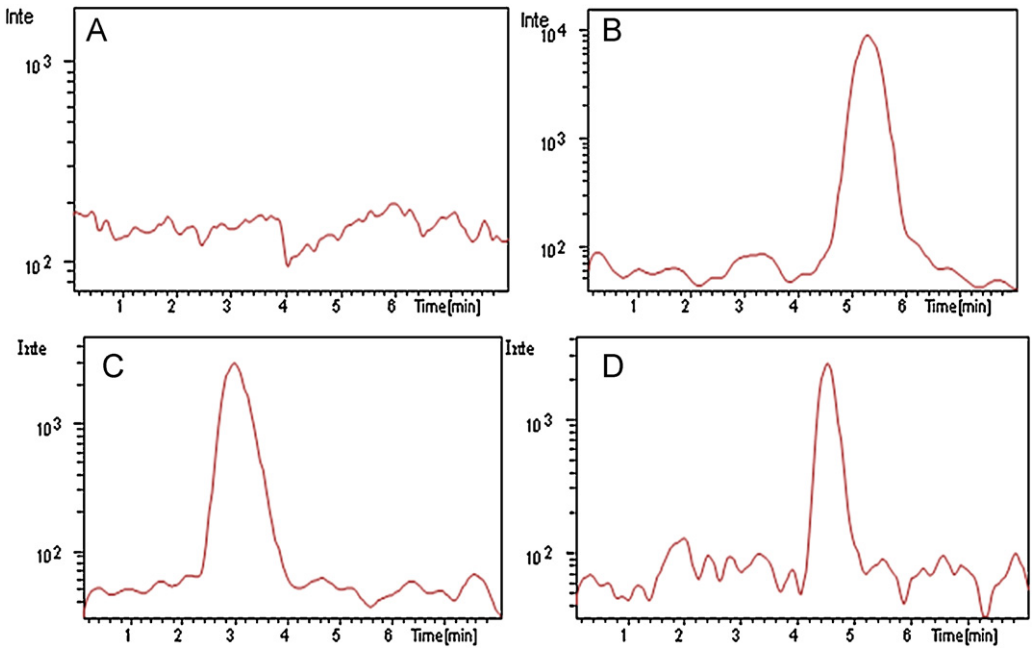

Fig. 3. Typical MRM Chromatograms of Blank(A), IS (B) and LLOQ chromatograms of LCA, 5 ng/mL (C) and LOS, 1 ng/mL (D).

Table 4
Stability data of LOS and LCA.

Sample type	Analyte	Conditions	Concentration taken (ng/mL)	Mean ± S.D.	% bias
Stock solution	LOS	0 h, RT	4	4.03 ± 0.16	−0.75
		24 h, RT		3.94 ± 0.20	−1.50
		0 h, RT	150	148.90 ± 3.17	−0.73
		24 h, RT		148.00 ± 2.92	−1.30
	LCA	0 h, RT	20	19.14 ± 0.45	−4.30
		24 h, RT		18.52 ± 0.86	−7.40
		0 h, RT	750	749.30 ± 6.85	−0.09
		24 h, RT		736.28 ± 15.73	−1.83
Whole blood	LOS	0 h, RT	4	3.96 ± 0.21	−1.00
		24 h, RT		3.73 ± 0.42	−6.75
		0 h, RT	150	148.62 ± 2.89	−0.92
		24 h, RT		146.85 ± 3.01	−2.10
	LCA	0 h, RT	20	19.26 ± 0.68	−3.70
		24 h, RT		18.21 ± 0.86	−8.95
		0 h, RT	750	747.30 ± 5.84	−0.36
		24 h, RT		729.14 ± 12.41	−2.78
DBS	LOS	30 days, RT	4		
		Day 1		3.98 ± 0.09	−0.50
		Day 10		3.69 ± 0.19	−7.75
		Day 20		3.73 ± 0.12	−6.75
		Day 30		3.56 ± 0.16	−11.00
		30 days, RT	150		
		Day 1		148.39 ± 4.12	−1.13
		Day 10		133.18 ± 3.19	−11.27
		Day 20		141.74 ± 3.67	−5.53
		Day 30		135.87 ± 3.98	−9.47
DBS	LCA	30 days, RT	20		
		Day 1		19.77 ± 0.51	−1.15
		Day 10		18.13 ± 0.57	−9.35
		Day 20		19.24 ± 0.87	−3.80
		Day 30		18.89 ± 0.84	−5.55
		30 days, RT	750		
		Day 1		745.15 ± 15.15	−0.65
		Day 10		738.91 ± 13.24	−1.48
		Day 20		741.65 ± 14.96	−1.12
		Day 30		740.85 ± 12.95	−1.23
Processed	LOS	0 h, 4 °C	4	4.00 ± 0.17	0.00
		24 h, 4 °C		3.89 ± 0.23	−2.75
		0 h, 4 °C	150	148.20 ± 2.78	−1.20
		24 h, 4 °C		148.30 ± 2.99	−1.13
	LCA	0 h, 4 °C	20	19.44 ± 0.50	−2.80
		24 h, 4 °C		18.57 ± 0.51	−7.15
		0 h, 4 °C	750	742.51 ± 11.73	−1.00
		24 h, 4 °C		740.00 ± 13.89	−1.33

Table 5
Influence of spot volume on precision and accuracy of the assay of LOS and LCA at LQC and HQC.

Analyte, conc (ng/mL)	LOS, 4			LOS, 150			LCA, 20			LCA, 750		
Volume spotted (μL)	10	15	20	10	15	20	10	15	20	10	15	20
Mean (n = 6)	3.81	4.14	3.97	140.49	145.90	146.52	18.26	18.63	19.00	719.29	734.27	742.23
S.D.	0.05	0.17	0.17	3.30	5.79	4.59	0.28	0.41	0.62	8.05	30.62	30.63
Precision (% CV)	1.32	4.11	4.34	2.35	3.97	3.13	1.55	2.25	3.26	1.12	4.17	4.13
Accuracy (% bias)	−4.67	5.58	−0.67	−6.34	−2.37	−2.32	−8.72	−6.87	−2.32	−4.10	−2.10	−1.04
Difference from 10 μL spot (%)		−9.72	−4.0		−3.61	−4.02		−1.85	−3.73		−2.00	−3.06

Table 6
Influence of hematocrit value on precision and accuracy of the assay of LOS and LCA at MQC.

Analyte	LOS 150 ng/mL			LCA 750 ng/mL		
Hematocrit %	20%	35%	50%	20%	35%	50%
	131.04	148.26	149.35	721.01	725.34	749.13
	129.87	139.51	150.87	702.21	736.48	763.41
	127.60	135.98	151.98	681.02	729.61	736.10
Mean (n = 3)	129.50	141.25	150.73	701.41	730.47	749.54
S.D.	1.75	6.32	1.32	20.00	5.62	13.65
% CV	1.35	4.47	0.87	2.85	0.76	1.82
% bias	−13.66	−5.83	0.48	−6.47	−2.60	−0.06
% difference from 35% Hct	7.83	0	−6.32	3.87	0	2.54

indicates that there was no significant difference in distribution of analytes and blood across spots derived from 15 and 20 μL. The experiment was further explored with smaller volume, i.e., 5 μL but assay accuracy and precision were found to be beyond the acceptable limits (>15%). Hence 10 μL was the minimum spot volume for determination of LOS and LCA by the proposed method.

3.3.9. Influence of hematocrit value

The tested analytes has shown a correlation with hematocrit value, it means the analyte concentrations were found to be significantly higher in samples with high hematocrit value and lower with low hematocrit value. The measured LOS and LCA concentration results were compared with the results obtained from the DBS samples with Hct of 35% and given in Table 6. The % difference was calculated by subtracting the % bias of Hct 20% and 50% from % bias of 35% Hct which was taken as standard Hct value. These results revealed that there was an apparent impact of Hct (−6.32 to 7.83%) on the quantification of LOS and LCA.

4. Conclusions

The present study describes for the first time a complete method validation for the simultaneous determination of LOS and LCA on dried blood spots. The novel bioanalytical method, employing DBS as sample collection technique with MS detection and selection of appropriate ionization technique (ESI) with polarity (−ve) played an important role in the method development. The proposed method was found to be linear, accurate and precise. The extraction procedure developed was more feasible than the other methods reported in literature. Good results were obtained in terms of sensitivity (LLOQ, 1.0 and 5.0 ng/mL for LOS and LCA, respectively), precision (% CV < 5.95%), accuracy (% bias from −2.8 to 1.5) and recoveries (LOS, 89.13–93.50 and LCA, 92.73–97.67). The method has shown good results with other validation parameters like carryover (<5%), matrix effect (CV > 6%) and dilution integrity (% CV, 5.96 and % bias, −2.74). Stability of LOS and LCA in the DBS samples, stock solution and processed samples was evaluated at various tested conditions. The obtained results (% CV < 8.15) show that the analytes were stable in tested conditions. As the analytes were stable on FTA cards under test conditions for at least 30 days, this method can facilitate pharmacokinetics of losartan. The influence of hematocrit value and spot volume showed no significant effect on precision and accuracy assay of LOS and LCA. The proposed method was simple, accurate and precise with minimal matrix and carry over effects and all the validation parameters were well within the accepted limits. The method may find application in preclinical, pharmacokinetic and toxicokinetics studies of losartan.

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