Hiten J. Shah[1, 2, 3]
Mohan L. Kundlik[1]
Nitesh K. Patel[1]
Gunta Subbaiah[1]
Dasharath M. Patel[2]
Bhanubhai N. Suhagia[3]
Chhagan N. Patel[2]

[1]Bioanalytical Laboratory, Torrent Pharmaceutical Limited, Bhat, Gandhinagar, Gujarat, India
[2]Shri Sarvajanik Pharmacy College, Mehsana, Gujarat, India
[3]L. M. College of Pharmacy, Ahmedabad, Gujarat, India

Original Paper

Rapid determination of losartan and losartan acid in human plasma by multiplexed LC–MS/MS

A rapid LC–MS/MS method has been developed and validated for the determination of losartan (LOS) and its metabolite losartan acid (LA) (EXP-3174) in human plasma using multiplexing technique (two HPLC units connected to one MS/MS). LOS and LA were extracted from human plasma by SPE technique using Oasis HLB® cartridge without evaporation and reconstitution steps. Hydroflumethiazide (HFTZ) was used as an internal standard (IS). The analytes were separated on Zorbax SB C-18 column. The mass transition [M–H] ions used for detection were m/z 421.0 → 127.0 for LOS, m/z 435.0 → 157.0 for LA, and m/z 330.0 → 239.0 for HFTZ. The proposed method was validated over the concentration range of 2.5–2000 ng/mL for LOS and 5.0–3000 ng/mL for LA with correlation coefficient ≥0.9993. The overall recoveries for LOS, LA, and IS were 96.53, 99.86, and 94.16%, respectively. Total MS run time was 2.0 min/sample. The validated method has been successfully used to analyze human plasma samples for applications in 100 mg fasted and fed pharmacokinetic studies.

Keywords: Human plasma / Losartan / Losartan acid / Multiplexed LC–MS/MS / Negative ion electrospray

Received: April 26, 2009; revised: July 6, 2009; accepted: July 7, 2009

DOI 10.1002/jssc.200900287

1 Introduction

Losartan potassium (2-butyl-4-chloro-l-[*p*-(*o*-1*H*-tetrazol-5-ylphenyl)benzyl]imidazole-5-methanol monopotassium salt) is an orally active non-peptide angiotensin II receptor antagonist [1]. It has been shown to be antihypertensive in humans [2]. LOS is metabolized in the body to a pharmacologically active carboxylic acid metabolite losartan acid (LA or EXP 3174) [3], which is about five times more potent and has longer elimination half-life ($t_{½}$) than LOS [4, 5]. Hence, it is essential for quantitative analysis of LOS and its active metabolite (LA) in human plasma for pharmacokinetic study.

HPLC–fluorescence [6] and HPLC–UV [7–10] analytical methods have been reported in literature for monitoring plasma level of LOS and LA. The reported HPLC methods were not specific enough compared with present method because of more specificity of MS/MS detector compared with fluorescence and UV detector. LC–MS/MS methods [11–15] have been reported to monitor LOS with LA with minimum 3.5 min run time per sample. The reported LC–MS/MS methods do not have any multiplex LC–MS/MS (two HPLC units connected to one MS/MS or parallel two column LC in conjunction with a conventional single-source electrospray mass spectrometer) method for quantification of LOS and LA in human plasma. Parallel analysis approach for achieving high throughput, either using multiple inlets in the mass spectrometer source or multiplexing LC units into one MS is well established. While the multiple inlets in the mass spectrometer allow a fourfold increase in the throughput, the sensitivity loss and potential cross-inlets contaminations are major drawbacks [16]. Multiplexing LC unit set-up enables to overcome the two shortcomings and demonstrates that when two LC units feed one MS, the run time is reduced by 50%. This approach has been successfully used for the analysis of drug candidates in plasma [17–22]. Therefore, in present work, two HPLC units connected with one MS/MS set-up are utilized to achieve high throughput.

We report a new validated method for quantification of LOS and LA in human plasma. The advantages of the present method are mentioned below:

Correspondence: Hiten J. Shah, Bioanalytical Laboratory, Torrent Pharmaceutical Limited, Village Bhat, Gandhinagar-382428, Gujarat, India
E-mail: hiten12345@hotmail.com, hitenshah@torrentpharma.com
Fax: +91-079-23969135

Abbreviations: APCI, atmospheric pressure chemical ionization; **CS,** calibration standard; **HFTZ,** hydroflumethiazide; **HQC,** high quality control; **IS,** internal standard; **LA,** losartan acid; **LLOQ,** lower LOQ; **LOS,** losartan; **LQC,** low quality control; **MQC,** medium quality control; **QC,** quality control; **RE,** relative error; **RT,** retention time; **SRM,** selected reaction-monitoring; **ULOQ,** upper LOQ

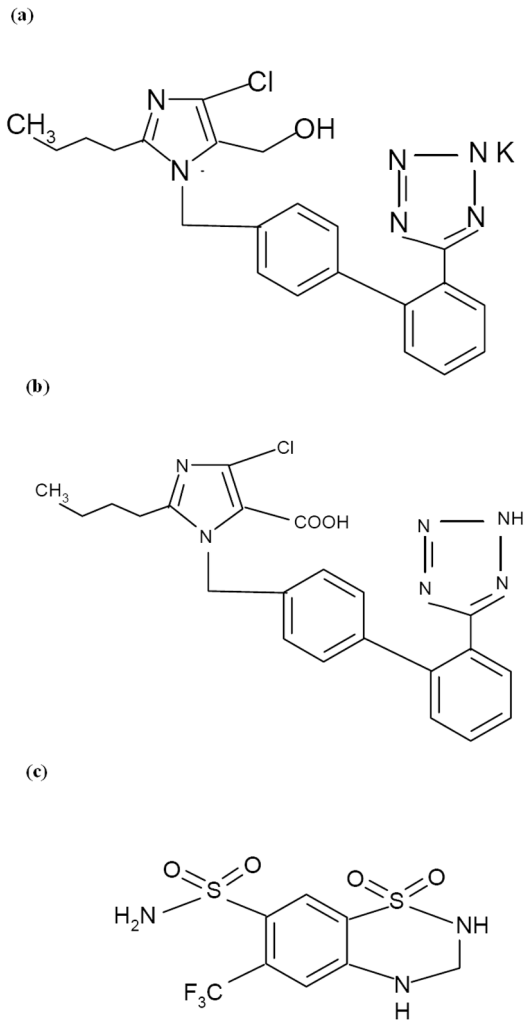

Figure 1. Chemical structures of (a) LOS, (b) LA, and (c) HFTZ.

(i) Save run time per sample (absolute 2.0 min/sample) with chromatographic separation and without any time gap in between two injections. Reported run times in minutes are 6 [11], 5 [12], 5 [13], 12 [14], and 3.5 [15]. Not a single reported method mentioned time between two injections.

(ii) Present method is simple and rapid SPE technique without drying and reconstitution steps. SPE methods with drying and reconstitution [11, 13], liquid–liquid extraction also with drying and reconstitution [12], and protein precipitation [14] methods have been reported in literature.

(iii) Current method has LLOQ (lower LOQ) 2.5 ng/mL and LOD 25 pg/mL for LOS and LLOQ 5 ng/mL and LOD 50 pg/mL for LA. In the literature [11, 12] 1 ng/mL LLOQ for both LOS and LA has been reported. However, Iwasa *et al.* [11] used 400 μL of human plasma.

(iv) Present method used only 200 μL of human plasma compare to reported 400 [11] and 500 μL [13] of human plasma, hence to reduce the amounts of blood withdrawn from volunteer during study.

(v) Recently reported [15] shortest run time method claimed to analyzed more than 200 samples/day while 700 samples/day analyzed by present method.

2 Experimental

2.1 Chemicals and reagents

Losartan (99.91%) and LA (99.83%) were obtained from Torrent Pharmaceutical Ltd. (Gandhinagar, India). Hydroflumethiazide (HFTZ) (97.00%) (Fig. 1) was procured from Sigma–Aldrich (Mumbai, India). Water was obtained using a Milli-Q water purification system from Millipore (Massachusetts, USA). Gradient grade methanol and ACN were purchased from Ranbaxy (Delhi, India). Suprapur® ammonia (25%) and orthophosphoric acid (85%) were obtained from Merck (Darmstadt, Germany).

2.2 Calibration curves

Nine-point standard calibration curves for LOS and LA were prepared by spiking the blank plasma with appropriate amounts of LOS and LA. The calibration curve ranged from 2.5 to 2000 ng/mL for LOS and 5.0 to 3000 for LA. Low quality control (LQC), medium quality control (MQC), and high quality control (HQC) samples were prepared at concentration levels of 7.5, 600, and 1400 ng/mL, respectively, for LOS and 15.0, 900, and 2100 ng/mL, respectively, for LA in plasma.

2.3 Sample preparation

A 200-μL aliquot of human plasma sample was mixed with 10 μL of internal standard (IS) working solution (2.0 μg/mL of HFTZ) followed by the addition of 200 μL of 5% orthophosphoric acid. The sample mixture was loaded onto an Oasis HLB® (1 cm^3, 30 mg) extraction cartridge that was pre-conditioned with 1.0 mL methanol followed by 2.0 mL water. Then the extraction cartridge was washed with 1 mL water followed by 1.0 mL 1% orthophosphoric acid and 1 mL 5% methanol. Subsequently, LOS, LA, and IS were eluted with 400 μL methanol/ACN (75:25 v/v). Then 5.0 μL of the extract was injected into the LC–MS/MS system without drying and reconstitution steps.

2.4 Instrumentation

The multiplexing set-up was consisted of two Shimadzu class VP series HPLCs (Fig. 2) and one flow change over

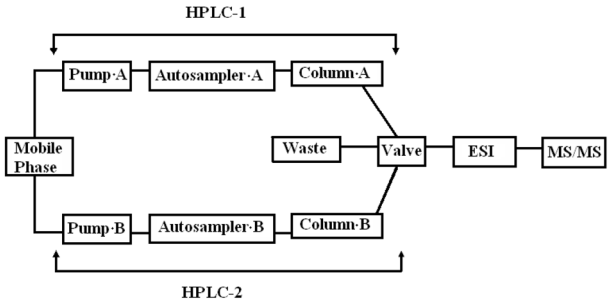

Figure 2. Multiplexing set-up.

Table 1. Ion source and analyte-dependent parameters of MS

Ion source			
Spray voltage	3200 V		
Capillary temperature	350°C		
Sheath gas	40 (Arbitrary)		
Auxillary gas	20 (Arbitrary)		
Polarity mode	Negative		
Analyte dependent	LOS	LA	IS
Precursor ion (*m*/*z*)	421.0	435.0	330.0
Product ion (*m*/*z*)	127.0	157.0	239.0
Tube lens off set (V)	95	74	94
Q1 Pw[a)] (amu)	0.5	0.5	0.5
Q3 Pw[b)] (amu)	0.5	0.5	0.5
Collision energy	42	41	32

[a)] Quadrapole 1 peak width
[b)] Quadrapole 3 peak width.

valve FCV-AH12 (Fig. 3). Chromatographic separation was carried out with Zorbax SB C18 (3.5 μm, 75 × 3.5 mm) column purchased from Thermo Electron Corporation, UK. A mobile phase consisting of water pH 9.0 with ammonia/ACN (20:80 v/v) was delivered with a flow rate of 0.3 mL/min without splitter. The column oven temperature was kept at 45°C. The total run time for each sample analysis was 3.2 min for both the HPLCs and 2.0 min for MS. The sample injection volume was 5.0 μL. Mass spectra were obtained using a TSQ Discovery mass spectrometer, a triple quadrupole mass analyzer with electron multiplier detector, equipped with ESI source (Thermo Finnigan Ltd., UK). The mass spectrometer was operated in the selected reaction-monitoring (SRM) mode. The MS parameters for analytes are listed in Table 1. Class-VP software (version 6.14 SP2) was used for HPLC function whereas MS data acquisition was ascertained by Xcaliber LCquan 2.5 software. The HPLC and MS data were synchronized by contact closure using a RS232 cable for communication and triggering. Within a single chromatographic run time in the multiplex LC–MS/MS, sample injections were made alternately onto each of two analytical columns in parallel at specified intervals, with a mass spectrometer data file opened at every injection. Thus, the mass spectrometer collected data from two sample injections into separate data files within a single chromatographic run time. Therefore, without sacrificing the chromatographic separation or the SRM, the sample throughput was increased by a factor of 2. Comparing the method validation results obtained using the two-column system with those obtained using the corresponding conventional single-column approach, the method on the two systems were found to be equivalent in terms of accuracy and precision.

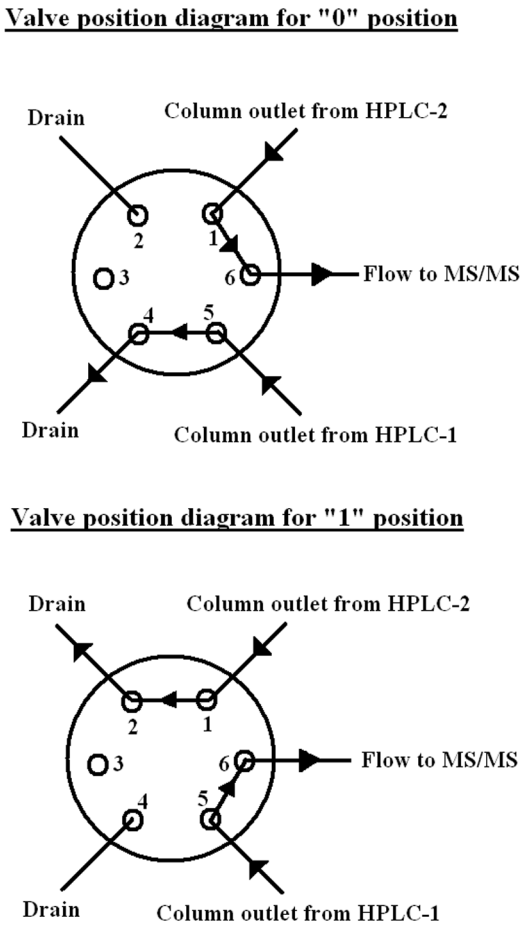

Figure 3. Flow change over valve.

2.5 Validation

The method was validated for selectivity, sensitivity, linearity, precision, accuracy, recovery, stability, matrix effect, and carry over check. Selectivity was performed by analyzing and comparing the blank plasma samples and spiked LLOQ samples from different sources (or donors) to ensure absence of any possible interference at the retention time (RT) of LOS, LA, and IS. Sensitivity was determined by analyzing six replicates of spiked plasma

with the analyte at LLOQ. The intra-run and inter-run accuracy were determined by replicate (n = 6) analysis of quality control (QC) samples and at LLOQ that were extracted from the same batch. Inter-run precision and accuracy of the calibration standards (CSs) were assessed using the six calibration curves used for assay validation.

The extraction efficiencies of LOS, LA, and IS were determined by comparing the peak area of extracted analytes to the peak area of equivalent non-extracted standards (drug spiked in extracted blank plasma) and equivalent aqueous standard.

The processed sample stability was evaluated by comparing the extracted plasma samples injected immediately (time 0), with the samples re-injected after keeping in the auto sampler at 5°C for 72 h. The stability of spiked human plasma stored at room temperature (bench-top stability) was evaluated for 12 h and compared with freshly prepared samples. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed five times, with freshly spiked QC samples. The stability of spiked human plasma stored at −70°C (long-term stability) was evaluated by analyzing LQC, MQC, and HQC samples that were stored at −70°C for 134 days together with freshly spiked CS and QC samples. All stability evaluations were based on back-calculated concentrations. Analytes were considered stable if the deviation of the mean test results were within 15% of freshly prepared or comparison sample.

Matrix effect was checked with total six different lots of plasma. Three samples each of LQC and HQC were prepared from different lots of plasma (in total 36 QC samples) and checked for the accuracy in all QC samples. This was performed to see the matrix effect of these different lots of plasma on the back calculated value of QCs *versus* their nominal concentrations. It is considered that there is no significant matrix effect if the deviation of the mean test results was within 15% of nominal concentration.

2.6 Application of method

The validated LC–MS/MS method was successfully applied in the pharmacokinetic studies of 100 mg losartan (LOS) tablets under fasting and fed conditions in healthy male volunteers of 36 ± 9 years in age and 65 ± 7 kg in weight. The design of the study comprised of a randomized, open label, single dose, two treatments, two periods, two sequence cross-over bioequivalence study of LOS 100 mg tablets in 14 healthy male volunteers under fasting and fed condition. Local ethical committee approved the study protocol. Each volunteer was judged to be in good health through medical history, physical examination, and routine laboratory tests. Written informed consent was obtained from each volunteer after detailed verbal and written information on the objective and the possible risks of the study. Volunteers were excluded if they had a history of smoking or drinking or if they were taking prescription medications within 14 days prior to the start of the study. The study was conducted inside Bioevaluation Centre, Torrent Pharmaceutical Ltd., Gandhinagar; strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA. A total of 1800 human plasma samples from 14 male volunteers for fast and 14 male volunteers for fed were analyzed along with CSs and QC samples in HPLC-1 and HPLC-2.

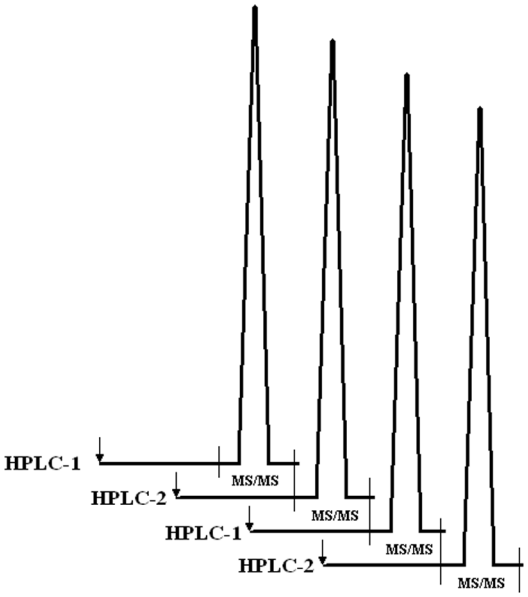

Figure 4. Diagram to explain relationships of injections and column-switching in multiplexed system. Arrow (↓) indicates injection from HPLC. Vertical lines in between MS/MS mark indicate the portion going to MS/MS and rest to waste. Vertical line is column switching time.

3 Results and discussion

3.1 Mass spectrometry

Losartan and LA donate the proton in a basic mobile phase and produce a negative precursor ion ([M−H]). Without multiplexed LC–MS/MS the RTs of LOS, LA, and HFTZ were 1.81, 2.17, and 2.41 min, respectively. It resulted in 1.2 min more comparing to the multiplexed RTs. Moreover, the total run time was 3.2 min/sample and in between two injections consumed 0.80 min/sample. Hence, the absolute run time was 4.0 min without multiplexed LC–MS/MS. While using multiplexed LC–MS/MS, no time is consumed in between two injections because after completion of injection from one HPLC, second HPLC is ready for injection. Hence absolute run time per sample is 2.0 min with multiplexed LC–MS/MS. Figure 4 shows the diagram to explain the relationships

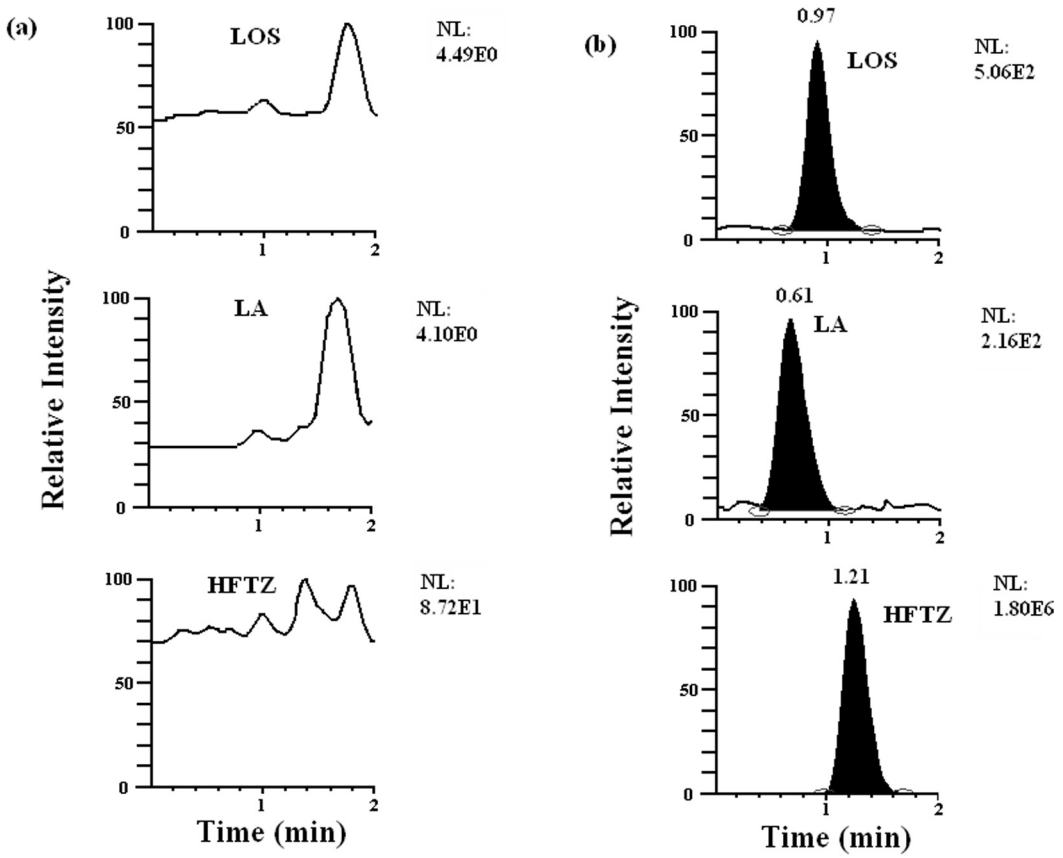

Figure 5. Chromatograms for blank plasma (drug and IS free), (b) chromatograms for LLOQ (2.5 ng/mL for LOS and 5.0 ng/mL for LA).

of injections and column-switching in multiplexed system. Further information on method development can be found online in the Supporting Information.

3.2 Method validation

The areas observed in six different lots of extracted blank plasma, at the RT of LOS and LA, were much less than 20% at the LLOQ area, whereas the area observed at the RT of IS was less then 5% the area of IS concentration used in sample preparation. Representative chromatograms of extracted blank plasma and extracted plasma samples containing 2.5 ng/mL LOS and 5.0 ng/mL LA (LLOQ) are presented in Figs. 5a and b, respectively.

Six linearity curves containing nine non-zero concentrations were analyzed (Supporting Information Table S1). The calibration curves appeared linear and were well described by least squares lines. A weighting factor of 1/concentration, *i.e.*, $1/x$ was chosen to achieve homogeneity of variance for LOS and LA. The correlation coefficients were ≥ 0.9993 ($n = 6$) for LOS and LA.

The LLOQ for LOS and LA were 2.5 and 5.0 ng/mL, respectively. The intra-run precision of the LLOQ plasma samples containing LOS were 3.41% and 3.27% for HPLC-1 and HPLC-2, respectively. The intra-run precision of the LLOQ plasma samples containing LA were 4.57% and 4.19% for HPLC-1 and HPLC-2, respectively. The mean intra-run accuracy of the LLOQ plasma samples containing LOS were 6.50% and 5.94% for HPLC-1 and HPLC-2, respectively. The mean intra-run accuracy of the LLOQ plasma samples containing LA were 6.98% and 6.84% for HPLC-1 and HPLC-2, respectively.

The intra-run precision ($n = 6$) was $\leq 7.32\%$ for HPLC-1 and $\leq 7.53\%$ for HPLC-2 for LOS furthermore $\leq 8.59\%$ for HPLC-1 and $\leq 8.77\%$ for HPLC-2 for LA. The intra-run accuracy was $\leq 8.71\%$ for HPLC-1 and $\leq 8.04\%$ for HPLC-2. The inter-run precision and accuracy were determined by pooling all individual assay results of replicate ($n = 6$) QC samples over the six separate batch runs. The inter-run precision was $\leq 8.31\%$. The inter-run accuracy was $\leq 6.14\%$ for LOS and LA.

Five replicates at LQC, MQC, and HQC concentrations for the LOS and LA were prepared for recovery determination. LOS mean absolute recovery was 96.53% with precision of 3.97%. LA mean absolute recovery was 99.86% with precision of 2.95%. The mean recovery for IS was 94.16% with precision of 3.46%. No significant difference (±2.68%) observed for absolute recovery and external

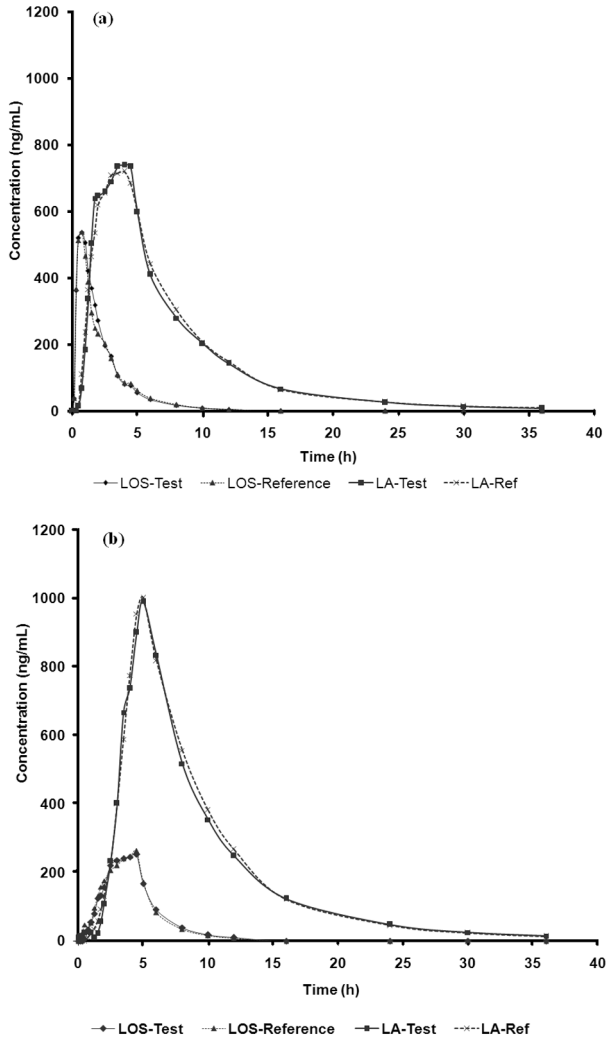

Figure 6. Representative data showing mean plasma concentration – time profiles of 14 healthy males after the administration of single oral dose of 100 mg of losartan potassium under (a) fasted and (b) fed condition.

spike sample in blank plasma eluent. Recoveries of the analytes and IS were consistent, precise, and reproducible.

All results of the stability studies were within acceptable criteria (Supporting Information Table S2).

For matrix effect three QC samples at each level along with the set of CSs were analyzed and the percentage bias of the samples analyzed was found within ±15% for each QC level for LOS and LA, proving that the elution of endogenous matrix peaks during the run has no affect on the quantification of LOS and LA (Supporting Information Table S3). Therefore, the method of extraction of LOS and LA from plasma was rugged enough and gave accurate and consistent results when applied to real study samples. In the earlier LC-MS/MS method published, the investigators pointed lower recoveries to suppression at the ion source; however, the matrix effect was not evaluated [11].

3.3 Application of method

One calibration curve was made for sample quantification on each HPLC-1 and HPLC-2. Total 700 samples were analyzed per day. The mean LOS and LA plasma concentration – time profile following 100 mg oral dose of LOS to human subjects is shown in Figs. 6a and b for fast and fed condition, respectively. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Supporting Information Table S4.

4 Concluding remarks

A simple, specific, rapid, and sensitive LC – MS/MS method has been developed for the determination of LOS and LA in human plasma. It is concluded that this sensitive and specific method is applicable for the high throughput quantitative determination of LOS in human plasma in pharmacokinetic, bioavailability, and *in vitro – in vivo* correlation studies of LOS.

The authors are indebted to Dr. Chaitanya Dutt, Director, Torrent Pharmaceutical Ltd., for his continuous support. The authors gratefully acknowledge Torrent Pharmaceutical Ltd. for providing necessary facilities to carry out this work.

The authors have declared no conflict of interest.

5 References

[1] Timmermans, P. B. M. W. M., Carini, D. J., Chiu, A. T., Duncia, J. V., Price, W. A., Wells, G. J., Wong, P. C., Wexlen, R. R., Johnson, A. L., *Am. J. Hypertens.* 1990, *3*, 599 – 604.

[2] Goldberg, A. I., Dunlay, M. C., Sweet, C. S., *Am. J. Cardiol.* 1995, *75*, 793 – 795.

[3] Lo, M. W., Goldberg, M. R., McCrea, J. B., Lu, H., Furtek, C. I., Bjornsson, T. D., *Clin. Pharmacol. Ther.* 1995, *58*, 641 – 649.

[4] Munafo, A., Christen, Y., Nussberger, J., Shum, L. Y., Borland, R. M., Lee, R. J., Waeber, B., Biollaz, J., Brunner, H. R., *Clin. Pharmacol. Ther.* 1992, *51*, 513 – 521.

[5] Lankford, S. M., Plummer, D., Hellyer, P., Christ, D. D., Bai, S. A., *J. Cardiovasc. Pharmacol.* 1997, *30*, 583 – 590.

[6] Ritter, M. A., Furtek, C. I., Lo, M. W., *J. Pharm. Biomed. Anal.* 1997, *15*, 1021 – 1029.

[7] Soldner, A., Langguth, H. S., Mutschler, E., *J. Pharm. Biomed. Anal.* 1998, *16*, 863 – 873.

[8] Yeung, P. K. F., Jamieson, A., Smith, G. J., Fice, D., Pollak, P. T., *Int. J. Pharma* 2000, *204*, 17 – 22.

[9] Zarghi, A., Foroutan, S. M., Shafaati, A., Khoddam, A., *Arzneim. Forsch.* 2005, *55*, 569 – 572.

[10] Bienert, A., Brzeziniski, R., Szalek, E., Dubai, V., Grzeskowiak, E., Dyderski, S., Drobnik, L., Wolc, A., Olejniczak-Rabinek, M., *Arzneim. Forsch.* 2006, *56*, 723 – 728.

[11] Iwasa, T., Takano, T., Hara, K., Kamei, T., *J. Chromatogr. B* 1999, *734*, 325 – 330.

[12] Michelle, P., Kerry, R., Song, H., Wai, L. M., *J. Pharm. Biomed. Anal.* 2003, *33*, 73 – 84.

[13] Oliveira, C. H., Medeiros, S. R., Santagada, V., Caliendo, G., Perissutti, E., Prado Galuppo, M., Marcondes, R. V., Barrientos-Astigarraga, R. E., Mendes, G. D., De, N. G., *Int. J. Clin. Pharmacol. Ther.* 2006, *44*, 142 – 148.

[14] Ferreiros, N., Dresen, S., Alonso, R. M., Weinmann, W., *Ther. Drug Monit.* 2007, *29*, 824 – 834.

[15] Budi, P., Lucy, S., Yahdiana, H., Windy, L., Matthew, G., *J. Pharm. Biomed. Anal.* 2009, *49*, 862 – 867.

[16] Yang, L., Wu, N., Rudewicz, P. J., *J. Chromatogr. A* 2001, *926*, 43 – 55.

[17] Korfmacher, W. A., Veals, J., Meynell, K. D., Zhang, X. P., Tucker, G., Cox, K. A., Lin, C. C., *Rapid Commun. Mass Spectrom.* 1999, *13*, 1991 – 1998.

[18] Van Pelt, C. K., Corso, T. N., Schultz, G. A., Lowes, S., Henion, J., *Anal. Chem.* 2001, *73*, 582 – 588.

[19] King, R. C., Stein, C. M., Magiera, D. J., Brann, J., *Rapid Commun. Mass Spectrom.* 2002, *16*, 43 – 52.

[20] Oertel, R., Richter, K., Fauler, J., Kirch, W., *J. Chromatogr. A* 2002, *948*, 187 – 192.

[21] Jemal, M., Huang, M., Mao, Y., Whigan, D., Powell, M. L., *Rapid Commun. Mass Spectrom.* 2001, *15*, 994 – 999.

[22] Bhatt, J., Subbaiah, G., Kambli, S., Shah, B., Patel, M., Saxena, A., Baliga, A., Nigam, S., Parekh, H., Yadav, G., *J. Chromatogr. B* 2007, *852*, 374 – 381.

