Simultaneous Determination and Pharmacokinetics of Metolazone, Losartan and Losartan Carboxylic Acid in Rat Plasma by HPLC–ESI–MS-MS

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For the first time, we developed and validated a highly sensitive, selective and rapid HPLC–ESI–MS-MS method for simultaneous quantification of metolazone (MET), losartan (LOS) and its metabolite losartan carboxylic acid (LCA) in rat plasma. After solid-phase extraction, the analytes and internal standard (irbesartan) were extracted from 100 µL plasma sample on an Agilent Poroshell 120, EC-C18 (50 × 4.6 mm, i.d., 2.7 µm) column using 5 µL injection volume with a total run time of 3 min. Acidified methanol/water mixture was used as a mobile phase. The parent → product ion transitions for MET (m/z 366.0 → 258.9), LOS (m/z 423.2 → 207.0), LCA (m/z 437.0 → 235.1) and IS (m/z 429.2 → 207.0) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ion mode. The method was found to be linear in the range of 0.05–250 for MET, 2–3,000 for LOS and 4–3,500 ng/mL for LCA. The method was validated with respect to selectivity, linearity, accuracy, precision, recovery and stability according to accepted regulatory guidelines. The described method was successfully applied to preclinical pharmacokinetic studies of analytes after an oral administration of mixture of MET (1 mg/kg) and LOS (10 mg/kg) in rats.

Introduction

Hypertension is now considered as a part of a complex syndrome of changes in cardiac and vascular structure and function (1). To achieve the target blood pressure mandated by current guidelines, a large majority of patients require simultaneous administration of multiple antihypertensive agents (2, 3). The rationale of combination therapy may be theoretically favored by the fact that multiple factors contribute to the hypertension and achieving control of blood pressure with a single agent that acts through one particular mechanism may be unrealistic (4).

The rationale for combining antihypertensive agents relates in part to the concept that the blood-pressure-lowering effect may be enhanced when two classes such as angiotensin II receptor blocker like losartan (LOS) and diuretic like metolazone (MET, Metoz-L®, Centaur Pharmaceuticals Pvt. Ltd., India) are co-administered.

MET, chemically known as (7-chloro-2-methyl-3-(2-methylphenyl)-4-oxo-2,3-dihydro-1Hquinazoline-6-sulfonamide), is a quinazoline diuretic. It acts primarily to inhibit sodium reabsorption at the cortical diluting site and to a lesser extent in the proximal convoluted tubule (5, 6).

LOS, chemically known as (2-butyl-4-chloro-L-[p-(o-1H-tetrazol-5-ylphenyl)b enzy1][imidazole-5 methyl monopotassium salt]), is an orally active non-peptide angiotensin II receptor antagonist (7). It has a more potent active metabolite losartan carboxylic acid (LCA), chemically known as 2-butyl-4-chloro-L-[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl[methyl]-1H-imidazole-5-carboxylic Acid (8–10).

Reduction in blood volume brought about by MET activates the renin angiotensin system (RAS). MET also causes decrease in serum potassium, as a result of its diuretic effects (11). Administration of LOS blocks the activation of the RAS and reverses the potassium loss associated with the diuretic. The combination of LOS and MET has an additive effect on blood pressure control, which is sustained for at least 24 h (12).

Different analytical methods are reported in literature for the determination of MET individually and combination with other drugs in biological matrix including HPLC (13–16), LC–MS-MS (17–20) and UPLC–MS-MS (21, 22). Further, different analytical methods have been described for determination of LOS and its metabolite LCA, alone or with combination of other drugs in biological matrix including HPLC (23–25) and LC–MS–MS (26–38).

Information from the reported methods reveals that some methods used for the determination of MET, LOS and LCA are suitable for the determination of a single analyte (MET or LOS) or for an analyte (LOS) with a metabolite (LCA). Although there is one HPLC method with fluorescence detection (39) has been reported for simultaneous determination of MET and LOS in combined tablet and spiked human plasma, this method is however limited to pharmaceutical preparation and failed to determine MET in human plasma. Also, the reported method did not quantify LCA in human plasma. We have also reported one HPLC and one HPTLC method for simultaneous estimation of LOS and MET, but the scope of that research was limited to analysis of these compounds in bulk drug and formulation (40, 41).

LC–MS–MS and UPLC–MS–MS were demonstrated to be superior to all the mentioned techniques in the literature for simultaneous quantification of drugs in biological matrix because of its selectivity, sensitivity, simplicity and high throughput (42). Simultaneous quantification of antihypertensive drugs in biological matrix is very important and necessary because majority of these drugs prescribed in combination to get better result and control blood pressure at the adequate level. To the best of our knowledge, till date there is no published work on simultaneous quantification of MET, LOS and LCA in rat plasma using LC–MS–MS. Therefore, it is essential to develop competent bioanalytical assays that could be used for routine measurement of these drugs simultaneously in biological matrix. Hence, the main objective of this work was to develop and validate a simple, sensitive and high-throughput HPLC–ESI–MS–MS method for...
simultaneous quantification of MET LOS and LCA in rat plasma and applied it in preclinical pharmacokinetic (PK) studies.

**Experimental**

**Chemicals and reagents**

MET (purity >99.70%) and LOS (purity >99.60%) were obtained from Centaur Pharmaceuticals Pvt. Ltd. (Mumbai, India). LCA (purity >99.80%) and Ibesartan (IS), 2-buty1-3-[(4-[2-(2H-tetrazol-5-yl)phenyl]phenyl][methyl]-1,3-diazaspiro[4.4]non-1-en-4-one (IS) (purity >99.90%) were obtained from Aristo Pharmaceuticals Pvt. Ltd. (Mandideep, India). HPLC grade methanol, formic acid and orthophosphoric acid (85%) were purchased from Merck Ltd. (Mumbai, India). Ultra-pure water (18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA). The HPLC mobile phase and sample aliquots were filtered through a 0.22-μm Nylon-66 filters (Aliagent Technologies, USA) before use. Oasis® HLB 30 mg/1 cc solid-phase extraction (SPE) cartridges were obtained from Waters (Minford, MA, USA).

**LC–MS-MS instrumentation**

The LC system consisted of an Agilent 1260 infinity HPLC system (Agilent Technologies, USA), which is equipped with a quaternary pump (G1311B), degasser (G1322A), Autosampler (G1329B) and a thermostated column compartment (G1316A). Chromatographic separation was carried on an Agilent Poroshell 120, EC-C18 (50 × 4.6 mm, i.d., 2.7 μm) column at ambient temperature. An isocratic mobile phase consisting of 0.1% formic acid in methanol and 0.1% formic acid in water (85:15, v/v) with a flow rate of 1 mL/min was used to separate the analytes and IS up to a total retention time of 3 min.

Mass spectrometric detection was performed using Agilent 6460 Triple Quadrupole mass spectrometer (Agilent Technologies), which was operated with Agilent G1948B electro spray ionization (ESI) source. The MS recordings were performed in the positive ion multiple reaction monitoring (MRM) with drying gas temperature (N₂), 300°C; gas flow, 8 L/min (N₂); nebulizer pressure, 45 psi; capillary voltage, 4,000 V. The optimized fragmentor voltage was set at 20, 15, 25 and 20 V for MET, LOS, LCA and IS, respectively. The collision energy was set at 5, 7, 15 and 15 V for MET, LOS, LCA and IS, respectively. The precursor/product ion pairs monitored were m/z 366.1 → 258.9, m/z 423.2 → 207.0, m/z 437.0 → 235.1 and m/z 429.2 → 207.0 for MET, LOS, LCA and IS, respectively. An Agilent MassHunter Workstation Software (Agilent Technologies) was used for all data acquisition, processing and storage.

**Standards and quality control samples preparation**

The standard stock solutions (1 mg/mL) of MET, LOS, LCA and IS were separately prepared in methanol. Working standard solutions of required concentration range of MET, LOS and LCA for calibration and controls were prepared by serial dilution in water/methanol (50:50, v/v; diluent). A 200 ng/mL IS working solution was prepared by diluting its stock solution with diluent. The calibration standards were prepared daily by spiking 20 μL of the appropriate standard working solutions to 100 μL blank rat plasma to provide the final concentrations of MET at 0.05–250 ng/mL, LOS at 2–3,000 ng/mL and LCA at 4–3,500 ng/mL. Quality control (QC) samples at low (LQC), medium (MQC) and high (HQC) QC levels were prepared at the concentrations of 0.15, 80 and 200 ng/mL for MET, 5, 400 and 2,500 ng/mL for LOS, and 10, 1,250 and 3,000 for LCA. All solutions described above were kept stored under refrigeration at 4°C.

**Sample preparation**

A 100-μL aliquot of rat plasma was thawed at room temperature. The plasma was spiked with 20 μL of IS (200 ng/mL) and 50 μL 5% orthophosphoric acid (pH 4.5) in a 2-mL polypropylene tube. The sample was briefly vortex-mixed and then centrifuged at 5,000 × g for 2 min. The sample mixture was loaded onto an Oasis® HLB (30 mg/1 mL) SPE cartridge that was preconditioned with methanol followed by water. Plasma was drained out under nitrogen pressure and extraction cartridge was washed with 1 mL water, 500 μL 1% orthophosphoric acid followed by 5% 500 μL methanol. The sample was eluted by passing 400 μL of mobile phase and transferred into an autosampler vial for injection. A 5-μL aliquot of the eluent was injected into the LC–MS–MS system for analysis.

**Method validation**

The developed method was validated for selectivity, linearity, accuracy, precision (intra-day and inter-day), recovery, matrix effect and stability, according to the Food and Drug Administration (FDA) guidelines for Bioanalytical method validation (43). Selectivity was used to investigate whether there were interferences at the retention time of analyte and IS. The selectivity was investigated by comparing the LC–MS–MS chromatograms of blank plasma samples from six different rats, blank plasma spiked with analytes at lower limit of quantitation (LLOQ) levels and IS at 200 ng/mL, and plasma sample obtained after the administration of LOS and MET. Matrix effect was evaluated by comparing the mean peak area of analytes spiked in blank extracted plasma samples (A) with the corresponding mean peak areas obtained by direct injection of standard solutions of analytes (B). Matrix effect = (A/B) × 100. For a method to be free from the relative matrix effect, the percentage of coefficient of variation (% CV) of normalized matrix effect should be less than 15% (44, 45). Matrix effects for the IS were also investigated. Recoveries of MET, LOS, LCA and IS were determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. Linearity, accuracy and precision were assessed by analyzing three validation batches. Each batch included a set of calibration standards and six replicates of LLOQ and QC (LQC, MQC and HQC) levels and was processed on three separate days. Accuracy was expressed as percent relative error (% RE) and the precision was evaluated by % CV. The linearity of each calibration curve was assessed by plotting the peak area ratio (y) of the analytes peak areas to the IS peak versus the corresponding concentration (x) of the analytes in the freshly prepared plasma sample. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of 1/x (x= concentration) weighting. The bench top, auto sampler, freeze–thaw and 90 days storage condition stability of all analytes and IS in spiked plasma samples were investigated. The stability of the analytes and IS in stock solution was also evaluated.

**PK study**

All animal studies described were approved by the institutional animal ethical committee prior to the study. Six young
and healthy male wistar rats weighing 250 ± 50 g were obtained from the Laboratory Animal Center, Department of Pharmaceutical Sciences and Technology, Mesra, Ranchi, and housed in an environmentally controlled room (temperature: 20 ± 2°C, humidity: 60 ± 5°C, 12 h dark/light cycle) for at least 7 days before the experiment. The rats were divided into two subgroups. The rodents were given a commercial rat chow and water ad libitum. Before oral administration of the mixture of MET at 1 mg/kg and LOS at 10 mg/kg dissolved in 0.1% carboxy methyl cellulose, the rats were fasted for 12 h, but allowed free access to water. Approximately 250 μL blood samples were collected into heparinized tubes via ocular puncture before drug administration and at 0.15, 0.25, 1, 2, 4, 8, 24 and 48 h in Subgroup 1 and at 0.15, 0.5, 1.5, 3.0, 6.0, 12.0 and 36 h in

Figure 1. Chemical structure and product ion mass spectra of (A) MET, (B) LOS, (C) LCA and (D) IS. This figure is available in black and white in print and in color at JCS online.
Subgroup 2 using sparse sampling in two subgroups (data provided in the supplementary material) after drug administration, and centrifuged at 6,000 rpm for 10 min to separate out plasma. These plasma samples were stored at $-80^\circ$C immediately prior to analysis.

Phoenix® WinNonlin® (version 6.3; Certara USA, Inc., St. Louis, USA) was used to analyze PK profile of MET and LOS, using non-compartmental methods, in rat plasma.

**Results**

**Optimization of LC–MS-MS conditions**

ESI technique operating in the MRM and positive ion mode was chosen to obtain optimum sensitivity and selectivity for the simultaneous quantification of MET, LOS, LCA and IS. The most sensitive and selective mass transitions were $m/z$ 366.1 $\rightarrow$ 258.9 for MET, $m/z$ 423.2 $\rightarrow$ 207.0 for LOS, $m/z$ 437.0 $\rightarrow$ 235.1 for LCA and $m/z$ 429.2 $\rightarrow$ 207.0 for IS, obtained after the optimization of collision energy. Chemical structures and Positive product ion mass spectra of MET, LOS, LCA and IS are shown in Figure 1.

As MET, LOS and LCA have significant differences in drug–plasma binding, physico-chemical properties, it was difficult to optimize a single extraction procedure for these analytes. Several extraction methods such as protein precipitation (PPT), liquid–liquid extraction (LLE) and SPE were tried based on the previously reported method for MET (15–20) and, LOS and LCA (28–39). Finally, SPE procedure was chosen to extract the analytes from plasma because it provides high extraction efficiency, good reproducibility and less interference of endogenous matrix components compared with PPT and LLE.

The feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate, trifluoroacetic acid and formic acid along with altered flow-rates (in the range of 0.4–1 mL/min) were tested for complete chromatographic resolution of analytes and IS. Finally, compounds were eluted up to a total retention time of 3 min using an isocratic mobile phase consisting of 0.1% formic acid in methanol and 0.1% formic acid in water (85:15, v/v) with at 1 mL/min flow, and the injection volume was 5 µL.

**Figure 2.** Representative MRM chromatogram of blank plasma samples: MET (A1), LOS (A2), LCA (A3) and IS (A4). Representative MRM chromatogram of a blank plasma sample spiked with MET at the LLOQ of 0.05 ng/mL (B1), LOS at the LLOQ of 2 ng/mL (B2), LCA at the LLOQ of 4 ng/mL (B3) and IS at 200 ng/mL (B4). Representative MRM chromatogram of a plasma sample of MET (C1), LOS (C2) and LCA (C3) obtained from a rat at 3 h after an oral administration of mixture of MET and LOS, and IS at 200 ng/mL (C4).
Method validation

Selectivity
There were no significant direct interferences of the endogenous substances of the blank plasma found at the retention times of the analytes (at LLOQ levels) and IS (at 200 ng/mL concentration). The retention times were 0.74, 1.29, 1.57 and 1.45 min for MET, LOS, LCA and IS, respectively (Figure 2).

Extraction recovery and matrix effects
The extraction recoveries and matrix effects were calculated by analyzing six replicates at LLOQ, LQC, MQC and HQC levels of MET, LOS and LCA. The matrix effect in plasma was found to be in the range of 89.85–90.20, 88.30–93.63 and 88.10–89.54% for MET, LOS and LCA, respectively. The extraction recovery was found to be in the range of 75.25–88.53, 91.29–93.20 and 88.53–90.90% for MET, LOS and LCA, respectively. The matrix effect and mean recovery for IS (at 200 ng/mL concentration) were found to be 92.90 and 93.60%, respectively.

Sensitivity and linearity
The LLOQ of the assay was 0.05, 2 and 4 ng/mL for MET, LOS and LCA, respectively. All three calibration curves were linear from 0.05 to 250, from 2 to 3,000 and from 4 to 3,500 ng/mL for MET, LOS and LCA, respectively. The calibration curve was created by plotting peak-area ratios of MET, LOS and LCA to IS versus their respective concentrations in rat plasma. Weighted (1/x) factor with a regression equation was used to determine the slope, intercept and correlation coefficient. All standard curves were linear ($r^2 \geq 0.9980$).

Precision and accuracy
The precision and accuracy of the developed method was determined by analyzing the analytes at LLOQ and three QC (LQC, MQC and HQC) levels for MET, LOS and LCA, and were found to be in acceptable limit. Precision was expressed as % CV and was found less than 15% at each concentration level of the nominal concentration. Accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as % RE. The intra-day and inter-day precision and accuracy results for MET, LOS and LCA are shown in Table I.

Stability
The stability of MET, LOS, LCA and IS in rat plasma was evaluated under the conditions described in Table II. All the analyzed samples were found to be stable in all cases as per the international acceptance criteria.

Application to PK study
The present method was applied successfully to the PK study of MET, LOS and LCA in wistar rats after an oral administration of the mixture of MET (1 mg/kg) and LOS (10 mg/kg). A representative chromatogram of post-dose sample (after 3 h) is shown in Figure 2. The resulting mean plasma concentration–time curves of MET, LOS and LCA are shown in Figure 3. The main PK parameters are summarized in Table III. This is the first study done on PKs of MET when given concomitantly with LOS in rats.

### Table I

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Nominal conc., ng/mL</th>
<th>Mean conc. found, ng/mL ± SD</th>
<th>Accuracya (% RE)</th>
<th>Precisionb (% CV)</th>
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<tbody>
<tr>
<td>MET</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-day (n = 6)</td>
<td>0.05</td>
<td>0.05 ± 0.01</td>
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<td>7.90</td>
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<td>Intra-day (n = 6)</td>
<td>80</td>
<td>79.95 ± 1.16</td>
<td>1.92</td>
<td>1.46</td>
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<tr>
<td>Inter-day (n = 6)</td>
<td>200</td>
<td>201.25 ± 2.09</td>
<td>-1.60</td>
<td>1.04</td>
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<tr>
<td>Inter-day (n = 6)</td>
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<td>0.508 ± 0.30</td>
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<td>3.30</td>
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<td>Inter-day (n = 6)</td>
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<td>80.85 ± 1.85</td>
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<td>1.46</td>
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<td>Inter-day (n = 6)</td>
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<td>202.25 ± 2.09</td>
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<td>1.25</td>
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<tr>
<td>LOS</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-day (n = 6)</td>
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<td>6.77</td>
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<td>Intra-day (n = 6)</td>
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<td>2.39</td>
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<td>Inter-day (n = 6)</td>
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<td>203.20 ± 2.09</td>
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<td>10.20</td>
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<td>Inter-day (n = 6)</td>
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<td>5.15 ± 0.50</td>
<td>1.72</td>
<td>6.80</td>
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<td>Inter-day (n = 6)</td>
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<td>405.15 ± 8.55</td>
<td>1.98</td>
<td>4.30</td>
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<td>Inter-day (n = 6)</td>
<td>2,500</td>
<td>2,602.30 ± 5.50</td>
<td>1.98</td>
<td>4.30</td>
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<tr>
<td>LCA</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Intra-day (n = 6)</td>
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<td>Intra-day (n = 6)</td>
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<td>Inter-day (n = 6)</td>
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<tr>
<td>Inter-day (n = 6)</td>
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<td>10.10 ± 0.60</td>
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<td>Inter-day (n = 6)</td>
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<td>3,155.70 ± 102.50</td>
<td>2.12</td>
<td>1.75</td>
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*% RE = (measured concentration – nominal concentration)/nominal concentration × 100.
% CV = SD of measured value/mean measured value × 100.

### Table II

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Nominal conc., ng/mL</th>
<th>Bench-top stability at 24 °C 90 Days</th>
<th>Long-term stability at −80 °C 90 Days</th>
<th>Auto sampler stability at 4 °C 24 h</th>
<th>Freeze–thaw stability from −80°C 24°C</th>
<th>Stock solution stability 4–8 °C 7 Days</th>
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<tr>
<td>MET</td>
<td></td>
<td>Conc. found, ng/mL ± SD</td>
<td>CV (%)</td>
<td>Conc. found, ng/mL ± SD</td>
<td>CV (%)</td>
<td>Conc. found, ng/mL ± SD</td>
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<tr>
<td>0.15</td>
<td>0.15 ± 0.20</td>
<td>3.87</td>
<td>0.15 ± 0.10</td>
<td>3.55</td>
<td>0.14 ± 0.15</td>
<td>3.90</td>
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<tr>
<td>80</td>
<td>80.15 ± 0.16</td>
<td>1.46</td>
<td>80.10 ± 0.16</td>
<td>1.46</td>
<td>79.99 ± 1.15</td>
<td>1.40</td>
</tr>
<tr>
<td>200</td>
<td>201.00 ± 2.09</td>
<td>1.04</td>
<td>201.00 ± 1.19</td>
<td>1.03</td>
<td>203.15 ± 2.15</td>
<td>1.09</td>
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<td>LOS</td>
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<td>5.01 ± 0.36</td>
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<td>400</td>
<td>403.117 ± 5.59</td>
<td>1.39</td>
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<td>401.15 ± 3.50</td>
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<tr>
<td>Inter-day (n = 6)</td>
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<td>2,515.33 ± 21.42</td>
<td>0.85</td>
<td>2,513.10 ± 15.50</td>
<td>0.80</td>
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<td>LCA</td>
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<td>10</td>
<td>10.15 ± 0.80</td>
<td>3.23</td>
<td>10.85 ± 0.65</td>
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<td>2,500</td>
<td>2,623.66 ± 18.87</td>
<td>1.49</td>
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<td>1.43</td>
<td>2,620.40 ± 14.50</td>
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<td>IS</td>
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<td>3,000</td>
<td>3,155.12 ± 22.50</td>
<td>3.31</td>
<td>3,151.5 ± 15.50</td>
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<tr>
<td>200</td>
<td>196.20 ± 5.10</td>
<td>2.29</td>
<td>196.13 ± 2.17</td>
<td>4.85</td>
<td>197.14 ± 3.12</td>
<td>2.64</td>
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</table>
C. LCA after an oral administration of MET (1 mg)

Several analytical methods are reported for quantitative determination of MET in biological matrices using HPLC with ultraviolet (UV), fluorescent or tandem mass spectrometric (MS–MS). These reported methods are having several disadvantages such as less sensitivity (23–25), long run time (23, 24), large volume of sample (26) and high matrix effect (26–29).

In this study, SPE method was found to be suitable to get maximum recoveries with minimum interference of endogenous plasma materials. The PK parameters evaluated in this method were also found similar to previously reported method (18, 33). The developed LC–MS–MS method has advantages as compare with previously reported method such as shorter analytical run time (3 min), high specificity (low matrix effect), excellent sensitivity (low LLOQ) for simultaneous estimation of MET, LOS and LCA in rat plasma.

**Discussion**

Several analytical methods are reported for quantitative determination of MET in biological matrices using HPLC with ultraviolet (UV), fluorescent or tandem mass spectrometric (MS–MS). These reported methods are having several limitation such as low recoveries and less sensitivity (13–15), longer retention time (15) and complex sample preparation (21, 22). Similarly, detection of LOS and LCA in various biological matrices by HPLC with UV, fluorescent or tandem mass spectrometric (MS–MS) detection has been reported but these methods also having several disadvantages such as low sensitivity (23–25), longer run time (23, 24), large volume of sample (26) and high matrix effect (26–29).

In this study, SPE method was found to be suitable to get maximum recoveries with minimum interference of endogenous plasma materials. The PK parameters evaluated in this method were also found similar to previously reported method (18, 33). The developed LC–MS–MS method has advantages as compare with previously reported method such as shorter analytical run time (3 min), high specificity (low matrix effect), excellent sensitivity (low LLOQ) for simultaneous estimation of MET, LOS and LCA in rat plasma.

![Figure 3. Mean plasma concentration versus time curve of (a) MET, (b) LOS and (c) LCA after an oral administration of MET (1 mg/kg) and LOS (10 mg/kg).](http://chromsci.oxfordjournals.org)

**Table III**

PK Parameters Summary—Combined Data From Two Subgroups

<table>
<thead>
<tr>
<th>PK parameter</th>
<th>Estimate (Mean ± SD, N = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (0–48 h), h × ng/mL</td>
<td>870.43 ± 38.84, 6,772.35 ± 598.26, 14,760.79 ± 1,829.82</td>
</tr>
<tr>
<td>AUC_{infty}, h × ng/mL</td>
<td>924.78 ± 40.33( ^* ), 6,804.34 ± 604.39, 14,810.80 ± 1,853.67</td>
</tr>
<tr>
<td>C_{max}, ng/mL</td>
<td>87.55 ± 13.40, 1,096.67 ± 182.58, 1,370.83 ± 156.86</td>
</tr>
<tr>
<td>T_{1/2}, h</td>
<td>0.07 ± 0.02( ^* ), 0.16 ± 0.03, 0.14 ± 0.01</td>
</tr>
<tr>
<td>T_{max}, h</td>
<td>9.40 ± 1.21, 5.54 ± 1.19, 7.04 ± 1.01</td>
</tr>
</tbody>
</table>

\( ^* N = 3. \)

References

1. Karla, S., Kalra, B., Agrawal, N.; Combination therapy in hypertension: an update; *Diabetology and Metabolic Syndrome*, (2010); 2: 44.

**Supplementary Material**


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**Conclusion**

In summary, for the first time a simple, rapid and cost-effective LC–MS–MS method was successfully developed and validated for simultaneous quantification of MET, LOS and LCA in rat plasma using single IS. Advantages of the current method are less sample volume, utilize simple extraction method and enables simultaneous estimation of combination drugs. The SPE elute (5 μL) directly injected to LC–MS–MS system without drying and reconstitution steps to give high throughput. The total elution time was 3 min. The developed method was sensitive enough and having low LLOQ of all three analytes to conduct a PK study. The developed LC–MS–MS method was validated for selectivity, linearity, precision, accuracy, recovery and stability, and was successfully applied to PK studies after an oral administration of MET (1 mg/kg) and LOS (10 mg/kg) in rats. These results indicate that simultaneous determination of MET, LOS and LCA is feasible in rat plasma. There is no common metabolism involved between MET and LOS so it seems that there are very less chances of any PK drug interaction between these two drugs but there can be PK interaction of these two drugs with other co-administered antihypertensive drugs. So established method can be useful for the simultaneous quantification of MET, LOS and LCA in rat plasma and also applicable to PK study. Perhaps, this method can be useful for PK assessment of the above-mentioned drugs in human plasma with proper modification.


44. Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M.; Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS; *Analytical Chemistry*, (2003); 75: 3019–3030.

45. Tudela, E., Munoz, G., Munoz-Guerra, J.A.; Matrix effect marker for multianalyte analysis by LC–MS/MS in biological samples; *Journal of Chromatography B*, (2012); 901: 98–106.