Determination of obeticholic acid and its glycine conjugate in human plasma using liquid chromatography-tandem mass spectrometry

Muralikrishna Ramisetti¹, Lakshmana Rao Atmakuri²*, Rama Shekara Reddy Dachuru³

¹Faculty of Pharmacy, Krishna University, Machilipatnam, Andhra Pradesh, India
²Vallabhaneni Venkatadri Institute of Pharmaceutical Sciences, Gudlavalleru, Andhra Pradesh, India
³Department of Chemistry, Krishna University, Machilipatnam, Andhra Pradesh, India

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ABSTRACT

A sensitive and selective liquid chromatography/tandem mass spectrometry method was recommended by the authors for the determination orally bioavailable farnesoid X receptor agonist obeticholic acid along with its glycine conjugate glycoobeticholic acid human plasma. Obeticholic acid_d5 and glycoobeticholic acid_d5 were used as internal standards, respectively. After extraction with a mixture of tert-butyl methyl ether and dichloromethane, samples were separated on a phenyl column with a mobile phase of 2mM ammonium acetate with 0.01% formic acid and methanol (15:85, v/v). Analysis was performed on an AB Sciex 4500 triple quadrupole mass spectrometer and data acquisition was performed by multiple reaction monitoring (MRM) in the negative ionization mode. Obeticholic acid and its conjugate were quantified in the linearity range of 0.50-100.00 ng/mL and the correction coefficients were ≥0.99 during the validation. Precision and accuracy in different days (inter-day) and single day (intra-day) were meeting the acceptance criteria specified in the recent US FDA bioanalytical method validation guidelines. A variety of stability experiments in neat samples and plasma samples were conducted and the results are meeting the acceptance criteria. A run time of 2.5 min for each sample made it possible to analyze a greater number of samples in a short time, thus increasing productivity. This method could be useful to quantitate obeticholic acid and glycoobeticholic acid in real clinical samples.

INTRODUCTION

Primary biliary cholangitis (PBC), an unusual autoimmune liver disorder described by inflammation and gradual weakening of bile flow, following in cholestasis and the ultimate enhancement of cirrhosis of the liver, end-stage liver disorder, and mortality (Edwards et al., 2017; Kaplan and Gershwin, 2005). Ursodeoxycholic acid (UDCA), is a drug endorsed for the healing of PBC. However, about 50% of the patients treated with UDCA shows inadequate response (Kuiper et al., 2010). Obeticholic acid (OCA) (Figure 1), a farnesoid X receptor agonist, suggested treating PBC in adult patients with PBC in combination with UDCA (Edwards et al., 2017; Smith and Pegram, 2017; Ocaliva, 2016). After oral administration of 10 mg multiple doses of OCA, the peak plasma concentration reaches in 1.5 hours (Smith and Pegram, 2017). OCA is conjugated with glycine or taurine in the liver forms
its conjugates of glycoobeticholic acid (G-OCA) or tauroobeticholic acid (T-OCA), respectively (Trivedi et al., 2016). OCA and its conjugates bind to human plasma protein greater than 99% (Ocaliva, 2018).

**Figure 1:** Chemical structures of OCA and G-OCA

As of today, very few analytical methods have been described for the estimation of OCA. (Ruyue et al., 2018) have described an LC-MS/MS procedure with an LLOQ of 5.0 ng/mL. Glycyrhretinic acid used as an internal standard. Later, (Li et al., 2019) described an LC-MS/MS procedure to estimate OCA in rat plasma. Both Authors have employed a liquid-liquid extraction method for extraction of OCA from plasma and effectively used to a pharmacokinetic analysis in rats. For bio availability and bioequivalence studies (BA/BE) of OCA, it is imperative to establish the in vivo concentrations of free OCA and its conjugate G-OCA. The published LC-MS/MS procedures (Narayanasamy et al., 2019; Ocaliva, 2016) are appropriate to preclinical studies and not be appropriate to apply for clinical studies. Likewise, the informed methods have estimated OCA only in rat plasma. As of now, no LC-MS/MS procedure has been described for the estimation of OCA and its conjugate G-OCA in human plasma. Thus, the authors intended to develop a sensitive and specific LC-MS/MS process for the concurrent estimation of OCA and G-OCA in human plasma. Deuterated compounds obeticholic acid_d5 (IS1) and glycoobeticholic acid_d5 (IS2) was used for the quantification of OCA and G-OCA, respectively.

**MATERIALS AND METHODS**

**Standards and chemicals**

The standards of OCA (98%), obeticholic acid_d5 (99%), and glycoobeticholic acid_d5 (99%) were obtained from the Cearsynth Limited (Mumbai, India), whereas G-OCA (96%), was taken from SimSon Pharma Limited (Mumbai, India). Water, methanol, and acetonitrile of LC-MS grade were bought from J.T Baker (Phillipsburg, USA). Likewise, formic acid and ammonium acetate were acquired from Fisher Scientific (Waltham, MA, USA). Ten individual control plasma heaps were bought from the local blood bank (Deccan’s Pathological Labs, Hyderabad, India).

**LC–ESI-MS/MS analysis conditions**

The enhanced mass spectrometer settings are itemized in Table 1. An AB Sciex 4500 (Foster City, CA, USA) mass spectrometer paired with HPLC system (Shimadzu Corporations, Kyoto, Japan) was utilized in the current study. A negative ionization acquisition was performed in mass spectrometer with an electrospray ionization source (ESI). Ammonium acetate (2mM) with 0.01% formic acid and methanol (15:85, v/v) was used as mobile phase to separate the analytes on a phenyl column (Zorbax SB Phenyl 100*4.6mm, 3.5µm) and pumped at 0.5mL/min. An extracted sample of 10µL was injected onto the column which was maintained 30±5°C.

**Calibrator and quality controls**

Two distinct stocks of each analyte were made in methanol to prepare calibrators (CC) and quality controls (QC) at a 1µg/mL concentration. Intermediate dilutions to a spike in plasma were made in a blend of water and methanol (40:60, v/v; diluent). The internal standards (ISs) stock solutions were also made in methanol. The working solution (500 ng/mL of IS1 and IS2) was also made in the diluent. The stocks were stored in a refrigerator maintained at 2–8 °C. A part of 10µL of working solution of analytes was added to 990µL of blank plasma to achieve final concentrations of 0.50, 1.00, 2.00, 5.00, 10.00, 20.00, 50.00, and 100.00 ng/mL for OCA and G-OCA for calibrators and at four QC concentration levels of 0.50 (LLOQ QC), 1.50 (LQC), 50.00 (MQC) and 80.00 (HQC) in plasma and stored at −70 ± 10 °C until use.

**Sample preparation procedure**

Analytes from plasma were extracted using a simple LLE procedure. An aliquot 100µL of thawed samples was mixed with 10µL of combined 500 ng/mL of IS1 and IS2 dilution briefly vortexed and mixed with 0.01% formic acid (50µL) and again vortexed. The samples were extracted with a mixture of tert-butyl methyl ether and dichloromethane (1mL, 90:10, v/v). The samples were mixed for 2 min and centrifuged (4000 rpm,5 min). 0.8mL of supernatant was evaporated at 40°C to dryness using nitrogen. The residue was reconstituted in the mobile phase (250µL) and injected.

**Assay validation**

Assay performance was conducted out as per existing US FDA (Food and Drug Administration, 2018) guidelines and published reports (Liu et al., 2019). The parameters studied during the validation are carryover test, selectivity, sensitivity, specificity, linearity, matrix effect, recovery, precision and accuracy, dilution integrity and stability.
Figure 2: Typical MRM chromatograms of OCA (left panel) and the IS (right panel) in human blank plasma (A) and an LLOQ sample along with IS (C)

Figure 3: Typical MRM chromatograms of G-OCA (left panel) and the IS (right panel) in human blank plasma (A) and an LLOQ sample along with IS (C)
Table 1: Tandem mass–spectrometer working conditions

| Compound       | Q1 mz/ (amu) | Q3 m/z (amu) | Dwell time (ms) | EP(V) | DP (V) | CXP (eV) | CE (V) |
|---------------|-------------|--------------|----------------|------|-------|----------|-------|
| OCA           | 419.2       | 401.0        | 100            | -10  | -120  | -12      | -50   |
| OCA-d5        | 424.1       | 405.0        | 100            | -10  | -120  | -12      | -50   |
| G-OCA         | 476.4       | 73.9         | 100            | -10  | -100  | -10      | -70   |
| G-OCA-d5      | 481.1       | 74.0         | 100            | -10  | -100  | -10      | -70   |

Source parameters

| Curtain gas (psi) | GAS1 (psi) | GAS2 (psi) | ESI source temp. (°C) | Collision gas (psi) |
|------------------|------------|------------|------------------------|---------------------|
| 40               | 50         | 50         | 550                    | 10                  |

Table 2: Within run and inter-run results for OCA and G-OCA

| Analyte | QC Conc.(ng/mL) | Conc. found, ng/mL (mean ± SD) | Precision (%) | Accuracy (%) | Conc. found, ng/mL (mean ± SD) | Precision (%) | Accuracy (%) |
|---------|-----------------|-------------------------------|---------------|--------------|-------------------------------|---------------|--------------|
| OCA     | 0.5             | 0.45 ± 0.03                  | 7.10          | 89.92        | 0.47 ± 0.04                  | 9.36          | 93.61        |
|         | 1.5             | 1.50 ± 0.03                  | 2.05          | 100.11       | 1.51 ± 0.03                  | 2.23          | 100.62       |
|         | 50.0            | 51.76 ± 1.44                 | 2.78          | 103.52       | 52.42 ± 1.52                 | 2.89          | 104.85       |
|         | 80.0            | 81.79 ± 2.01                 | 2.46          | 102.24       | 82.68 ± 2.13                 | 2.58          | 103.35       |
| G-OCA   | 0.5             | 0.49 ± 0.05                  | 10.07         | 97.78        | 0.51 ± 0.07                  | 13.71         | 102.37       |
|         | 1.5             | 1.43 ± 0.02                  | 1.12          | 95.43        | 1.43 ± 0.03                  | 2.06          | 95.04        |
|         | 50.0            | 52.27 ± 1.29                 | 2.46          | 104.54       | 52.01 ± 1.16                 | 2.22          | 104.01       |
|         | 80.0            | 75.91 ± 2.42                 | 3.19          | 94.89        | 76.09 ± 2.09                 | 2.74          | 95.12        |

Table 3: Stability data for OCA and G-OCA (n=6)

| Test                                  | Conc.ng/mL | OCA | Mean±SD (ng/mL) | CV (%) | %Stab. | Mean±SD (ng/mL) | CV (%) | %Stab. |
|---------------------------------------|------------|-----|----------------|--------|--------|----------------|--------|--------|
| Stability at room temp. for 10 h       | 0.5        |     | 1.51±0.01      | 0.67   | 100.64 | 1.57±0.03      | 2.04   | 104.88 |
| Freeze thaw stability at -80°C (5 cycles) | 80.0      |     | 82.87±0.49      | 0.59   | 103.59 | 78.76±0.85      | 1.08   | 98.45  |
| Processed samples stability (at 2-8°C for 46h) | 80.0    |     | 85.45±1.14      | 1.33   | 106.81 | 79.35±0.93      | 1.18   | 99.19  |
| Autosampler stability (at 5°C for 58h) | 80.0      |     | 85.45±0.79      | 0.93   | 106.84 | 80.47±1.23      | 1.52   | 100.59 |
| Reinjection stability (63h)           | 80.0      |     | 81.28±0.91      | 1.12   | 101.60 | 82.10±0.92      | 1.12   | 102.62 |
| Long-term Stability in plasma (at-80°C for 60 days) | 80.0  |     | 83.71±1.35      | 1.61   | 104.63 | 78.02±1.35      | 1.73   | 97.52  |
RESULTS AND DISCUSSION

Method development

The intention of the suggested study was to develop a sensitive LC-MS/MS method for the estimation of OCA and G-OCA in plasma proper for pharmacokinetic/toxicokinetic and bioequivalence evaluation of newer formulations. To attain the aim, several choices were assessed comprising tuning of mass spectrometry settings, selection of chromatography, and sample preparation. A 100 ng/mL solution of analyte was introduced into the ESI chamber of the mass spectrometer in negative ion mode. The ion spray voltage and decluttering potential were adjusted to attain intense and stable parent spectra of analytes. Additional, collision energy, collision cell exit potential, and collision gas were appropriately adjusted to achieve stable and intense production (Q3 MS) spectra. Likewise, the source parameters, namely source temperature and curtain gas, were adjusted to enhance the intensity of OCA and G-OCA. Dwell time of 100 ms was set for each compound. The most sensitive m/z values were obtained at 419 to 401 for OCA, m/z 424 to 405 for IS1, m/z 476 to 74 for G-OCA, and from m/z 481 to 74 for IS2. MRM technique was applied to quantify the analytes. The studies reported (Narayanasamy et al., 2019) that MRM technique provides the intrinsic selectivity and sensitivity for many compounds, hence applied to quantify the analytes in the present study.

Extensively used organic solvents in LC-MS analysis are acetonitrile and methanol. Similarly, ammonium acetate and ammonium formate are commonly used volatile buffers along with acid modifiers formic acid and acetic acid. Method development started using acetonitrile as an organic solvent, but acetonitrile combined with above buffers gave an insufficient response to quantify OCA and G-OCA. Hence, acetonitrile was replaced with methanol. Methanol, in combination with ammonium acetate (2 mM), gave good peak shape with the highest response for both analytes. Mixing of formic acid to the buffer solution attained higher detection levels for OCA and G-OCA. Among the different columns tested, Zorbax SB Phenyl (100×4.6mm, 3.5 μm) offered good peak shape and response for the analytes. The mobile phase was pushed at a flow rate of 0.5 mL/min.

The sample cleanup is very important in LC-MS analysis as it was significantly impacting the mass spectrometry ionization. As aimed to get effective extraction with high recoveries of analytes, LLE with a numerous solvent was tested. Those are ethyl acetate, tert-butyl methyl ether, diethyl ether, hexane, and dichloromethane. Also, a combination of various solvent tested. Favorable results were achieved with a combination of tert-butyl methyl ether and dichloromethane (90:10, v/v). The mean recoveries of >80% for OCA and G-OCA. Addition of formic acid (0.01%) during extraction helped obtain high recoveries for OCA and G-OCA. Deuterated standards OCA-d5, and G-OCA-d5, were utilized as ISs, to quantitate OCA and G-OCA, respectively.

Method validation

LC-ESI-MS performance was monitored each day before setting up the analysis. For system suitability experiment, a neat sample of the analytes was injected (n=6) into LC-ESI-MS system. The precision for system suitability assessment was observed to be less than 2% for the area ratio of OCA and G-OCA. Injector carryover test was performed during the method validation. A mixture of water and methanol (50:50, v/v) was used to rinse the injector after each injection. No carryover was identified in the blank sample after injection of maximum concentration of each analyte. Six specific plasma batches (4 of K2EDTA, 1 hemolyzed and 1 lipemic) were examined for selectivity experiment. No interference was found in all the plasma lots tested. A representative chromatogram of OCA and G-OCA of the blank sample, blank sample with IS and an LLOQ sample are shown in Figures 2 and 3, respectively. The selected internal standards did not interfere with the analytes. A sensitivity test was conducted to determine the lower limit of quantification (LLOQ) and it was established at 0.50 ng/mL for OCA and G-OCA. The precision and accuracy results at LLOQ concentration were 5.54 and 86.37% and 12.65 and 92.93% for OCA and G-OCA. The S/N or signal-to-noise ratio at LLOQ concentration was ≥5 for both analytes.

A regression equation with 1/x^2 weighting factor found best fit for the present method in the calibration 0.50-100.00 ng/mL for OCA and G-OCA in plasma. The correlation coefficient (r^2) was ≥0.98 for both analytes. A total of three precision and accuracy runs were made throughout the validation. Each run comprises a blank sample, blank with the ISS, 9 nonzero concentrations and 6 sets of QC at each concentration level. The resulted within-run and between-runs precision and accuracy data are presented in Table 2. Matrix effect was assessed by determining IS normalized matrix factor in 6 plasma batches. Post extraction spiked samples were complemented with neatly prepared in the mobile phase. IS normalized matrix factor at LQC and HQC level was 1.10 and 0.95 for OCA and 1.08 and 0.95 for G-OCA.

Similarly, the precision (%CV) at LQC and HQC level
was less than 5% for both the drugs, indicating no significant ion suppression and enhancement on the ionization of OCA and G-OCA. Recoveries with LLE as sample preparation were high and consistent. The mean overall recovery of OCA and G-OCA were 85.08 and 81.16%, with a precision range of 0.88-5.43% and 0.74 to 5.34%, respectively. Similarly, the recovery of the IS1 and IS2 was 81.18 and 78.01%. The in vivo sample concentrations above the ULOQ (100.00 ng/mL of OCA and G-OCA) can be diluted with the screened blank matrix. The upper limit of quantitation limit of OCA and G-OCA can be extended to 300.00 ng/mL (3 times of ULOQ) by using 1:4 dilution with blank plasma. The precision and accuracy for OCA at 1/4 dilution were observed to be 0.89 and 98.27%, respectively. Likewise, the precision and accuracy of G-OCA at 1/4 dilution were observed to be 1.79 and 95.67%, respectively.

The stability of OCA and G-OCA was evaluated under different conditions. The stock solution of OCA and G-OCA were stable for 52 days (at 2–8°C). The % stability (with %CV) of OCA and G-OCA was 97.11% (1.13 -1.98%) and 96.32% (0.45 -2.22%), respectively. Plasma sample stability after storage at −70°C in the freezer was evaluated after 60 days. Similarly, spiked samples were stable at room temperature for a minimum of 10 h. Processed samples were stable in auto sampler for 58 h and 46 h in the refrigerator at 2–8°C. Similarly, samples were stable for 63 h after reinjection. All the stabilities were evaluated at LQC and HQC levels (Table 3).

CONCLUSIONS
It was concluded that the authors recommended a simple, specific, and sensitive LC–ESI-MS/MS method for the measurement of OCA and G-OCA. As far as we know, this is the first LC-MS/MS method defines the comprehensive methodology for the concurrent measurement of OCA and G-OCA in human plasma. The procedures were validated as per the current US FDA guidelines. Deuterated standards were utilized as internal standards to reduce the matrix effect and recovery inconsistency between the analyte and the IS. Sample preparation with LLE gave highest and consistent recovery for the analytes. Overall, the present method showed outstanding performance with no matrix effect for the determination of OCA and G-OCA suitable for pharmacokinetic, toxicokinetic, bioavailability and bioequivalence studies in humans.

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Conflict of Interest
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