Determination of cyclovirobuxine D in human plasma by liquid chromatography tandem mass spectrometry and application in a pharmacokinetic study

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\textbf{Abstract} A sensitive and reliable method based on liquid chromatography tandem mass spectrometry (LC–MS/MS) for the quantitation of cyclovirobuxine D in human plasma has been developed and validated. Sample preparation by solid phase extraction was followed by separation on a CN column with a mobile phase of methanol–water (95:5, v/v) containing 0.2% formic acid. Mass spectrometric detection in the positive ion mode was carried out by selected reaction monitoring (SRM) of the transitions at $m/z$ 403.0 → 372.0 for cyclovirobuxine D and $m/z$ 325.0 → 234.0 for citalopram (internal standard). The method was linear in the range 10–200 ng/L with LLOQ of 10 ng/L, recovery >85%, and no significant matrix effects. Intra- and inter-day precisions were all <9% with accuracies of 94.0–104.8%. The method was successfully applied to a pharmacokinetic study involving a single oral administration of a 2 mg cyclovirobuxine D tablet to twenty-two healthy Chinese volunteers.

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1. Introduction

Cyclovirobuxine D (CVB-D; Fig. 1) is a steroidal alkaloid extracted from the plant Baux microphylla. It is widely used in China for the treatment of cardiac insufficiency, angina pectoris and arrhythmias. CVB-D can significantly increase the viability of cardiomyocytes injured by oxidation or hypoxia via cytoprotection, opening K\textsubscript{ATP} channels, stimulating NO release and inhibiting venous thrombosis\textsuperscript{2}.

CVB-D is orally active and requires a daily dose of only 2 mg for adults. This low dose leads to a very low concentration of CVB-D in body fluids making it difficult to carry out therapeutic drug monitoring (TDM) or pharmacokinetic studies. In fact, only a few methods for the determination of CVB-D in human body fluids have been reported despite the fact that CVB-D has been in clinical use for over twenty years.

Recently Yu et al.\textsuperscript{3} developed an analytical method based on liquid chromatography tandem mass spectrometry (LC–MS/MS) with electrospray ionization (ESI) and applied it to a pharmacokinetic study in healthy volunteers given 2 mg CVB-D by venous infusion. Despite a lower limit of quantitation (LLOQ) of 200 ng/L\textsuperscript{3}, the method remained unsuitable for the pharmacokinetic study because the C\textsubscript{max} of CVB-D was <200 ng/L. In the same year, Ding et al.\textsuperscript{4} reported an LC–MS method using atmospheric pressure chemical ionization (APCI) in which an LLOQ of 10.1 ng/L was claimed. Unfortunately the assay was performed on a single-quadrupole mass spectrometer with a highly aqueous mobile phase making the sensitivity and selectivity of the method rather doubtful. In addition, matrix effects were not evaluated and concentrations of CVB-D in all samples were within the lower 1/30 of the linear range. Moreover, both these methods involved liquid–liquid extraction (LLE) from basified samples, which resulted in significant adsorption of CVB-D onto glassware.

In order to establish a sensitive and reliable method for TDM and pharmacokinetic studies of CVB-D after oral administration of therapeutic doses, we have developed and validated a novel method based on sample preparation by solid phase extraction (SPE) followed by LC–MS/MS. The method overcomes the shortcomings of previous assays and has been successfully applied to a pharmacokinetic study of CVB-D tablets in healthy Chinese volunteers.

2. Materials and method

2.1. Materials

The CVB-D reference standard (batch number: 110888-200202, purity: 97.6\%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Citalopram, for use as internal standard (IS), was purchased from Sigma-Aldrich (St. Louis, USA). CVB-D tablets (1 mg formation) were provided by Tianjin LB Bioengineering Co., Ltd. (Tianjin, China). Deionized water was processed through a Milli-Q water purification system (Millipore, Milford, USA). Methanol was HPLC grade from Merck, Darmstadt, Germany. SPE columns (StrataTM-X 8B-S100-TAK) were purchased from Phenomenex (Torrance, USA) and mounted on a VacElut vacuum manifold (Supelco, Bellefonte, USA). Blank human plasma was obtained from the Nanjing Red Cross Blood Center (Nanjing, China). All other chemicals were of analytical grade and used as received.

2.2. Preparation of calibration standards and quality control samples

A CVB-D stock solution (100 mg/L) was prepared in methanol containing 0.2\% formic acid and stored at 4 °C when not in use. Standard solutions were made by dilution of the stock solution with methanol containing 0.2\% formic acid. Aliquots (20 \textmu L) of these standard solutions were used to spike 0.5 mL aliquots of blank human plasma to give calibration standards with concentrations of 0, 10, 20, 40, 75, 125 and 200 ng/L. Quality control (QC) samples (20, 75 and 150 ng/L) were prepared in the same way from an independently prepared stock solution. A working IS solution (200 ng/mL) in methanol was also prepared.

2.3. Sample preparation

Prior to SPE, frozen plasma samples were thawed in a water bath at 37 °C. SPE columns were activated by elution with 1 mL methanol followed by 1 mL water. Plasma samples (0.5 mL) were mixed with 0.5 mL of 20 mmol/L acetate buffer (pH 5.2) and 20 \textmu L IS working solution and loaded onto activated SPE columns. After elution at 1 mL/min under reduced pressure, columns were washed with 1 mL water followed by 1 mL 25\% methanol and dried under reduced pressure for 3 min. Finally, the analyte was eluted with 1 mL methanol containing 1\% formic acid, and the eluent evaporated to dryness under a gentle stream of nitrogen at 60 °C. Dried extracts were reconstituted in 100 \textmu L mobile phase and 10-\textmu L aliquots injected into the LC–MS system.

2.4. LC–MS/MS conditions

Analysis was performed on a Finnigan TSQ LC–MS/MS system (Thermo Electron Corporation, San Jose, USA) consisting of a Finnigan Surveyor LC pump, a Finnigan Surveyor refrigerated autosampler, and a Finnigan TSQ Quantum Ultra AM triple quadrupole tandem mass spectrometer with an APCI interface and a two-port diverter valve. The
LC–MS/MS system was operated under Xcalibur® 1.1 software at ambient temperature (25 ± 2 °C).

Chromatography was performed on a Phenomenex CN column (5 μm, 150 mm × 2.1 mm i.d., Torrance, USA) protected by 4.0 mm × 2.0 mm i.d. Phenomenex CN guard column. The isocratic mobile phase was methanol–water (95:5, v/v) containing 0.2% formic acid at a flow rate of 0.25 mL/min. The eluate was directed to waste for the first 2.8 min and then to the mass spectrometer using a diverter valve.

The APCI source was operated in the positive ion mode and optimized by constant infusion of a CVB-D standard solution (500 μg/L) in mobile phase into the HPLC flow using a syringe pump.

Figure 2  Representative chromatograms obtained from human plasma samples: (a) blank human plasma; (b) plasma sample spiked with CVB-D at 20 ng/L and citalopram (IS) at 40 ng/L; (c) a clinical sample.
2.5. Assay validation

Specificity was assessed by analysis of plasma samples from six individual donors. Linearity was tested by linear regression of calibration curves based on peak area ratios of analyte to IS. Evaluation of precision and accuracy involved analysis of five replicates of QC samples on three separate occasions. Recovery was determined by comparing peak areas of QC samples prior to extraction with those of plasma samples spiked post-extraction. Matrix effects were evaluated at 20, 75 and 150 ng/L for analyte by comparing the peak areas of samples (n=5) spiked post-extraction with those of standard solutions at the same concentration.

Stability of CVB-D was evaluated by assay in triplicate of QC samples under the following conditions: At ambient temperature over 12 h (short-term stability); at ~20 °C for over 30 days (long-term stability); and after three freeze–thaw cycles (room temperature to ~20 °C) (freeze–thaw stability). Post-preparative stability was also evaluated under autosampler conditions (10 °C) for over 24 h.

2.6. Pharmacokinetic study

A pharmacokinetic study in twenty-two healthy Chinese male volunteers was performed according to the Declaration of Helsinki for Biomedical Research involving human subjects and the rules of good clinical practice (GCP). All volunteers gave written informed consent before entering the study. Venous blood samples (5 mL) were collected into heparinized tubes before the dose and at 5, 15, 30, 45 min, 1, 2, 4, 10, 16, 24, 36, 48, 72, 96, 120 h after administration of a single oral dose of 2 mg CVB-D tablets, respectively. Blood samples were immediately centrifuged and plasma frozen at −20 °C until analysis. Pharmacokinetic parameters of CVB-D, including maximum plasma concentration (C_{max}), time to C_{max} (T_{max}), area under the plasma concentration–time curve from time zero to the last measurable concentration (AUC_{0–t}), area under the plasma concentration–time curve from time zero to infinity (AUC_{0–∞}) and elimination half-life (t_{1/2}), were calculated using DAS Ver 2.0 software.

3. Results and discussion

3.1. LC–MS conditions

Assay development in this study was based on previously reported methods. Thus chromatography was performed on a CN analytical column using a mobile phase containing a high percentage of organic solvent as described by Yu et al. This provided good ionization efficiency and elimination of ion suppression. An APCI source was employed as in the study by Ding et al. since it provided a more stable response than an ESI source. These features together with detection by MS/MS gave an LLOQ of 10 ng/L with good precision and accuracy. This sensitivity was suitable for TDM and for clinical pharmacokinetic studies.

3.2. Assay validation

3.2.1. Specificity

Representative chromatograms of blank and spiked human plasma samples are shown in Fig. 2. The mean retention times for CVB-D and IS were 4.9 and 3.3 min, respectively, with an overall chromatographic run time of 6 min. All plasma samples were free of interference at the retention times of both CVB-D and IS.

3.2.2. Linearity and LLOQ

The calibration curve was linear in the range 10–200 ng/L with r^2=0.99. The LLOQ (defined as the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy) was shown to be 10 ng/L on the basis of intra- and inter-day precision values of 6.27% and 5.73%, respectively, and corresponding accuracy values of 104.8% and 98.3%, respectively.

3.2.3. Precision and accuracy

Intra- and inter-day precision and accuracy results are summarized in Table 1. As shown, precision at all concentrations was <9% with accuracy in the range 94.0–104.8%. All values were within the limits recommended for bioanalytical method validation.

3.2.4. Sample preparation

CVB-D is a basic steroidal alkaloid, which has a tendency to adsorb to glassware under alkaline conditions. In previous methods, samples have been strongly basified and subjected to liquid–liquid extraction (LLE) with organic solvents. In order to reduce the uncertainty caused by adsorption, it was decided to avoid LLE and use SPE instead. After initial screening of SPE sorbents, StrataX columns were chosen because, unlike silica-based reversed phase columns, they are known to retain moderately polar compounds whilst allowing efficient washing to remove contaminants. In this study they produced clean extracts with high recovery of CVB-D. In fact, the extraction recoveries at concentrations of 20, 75 and 150 ng/L were 91.2%, 94.0% and 95.5%, respectively. Recovery of the internal standard was 94.8%.

3.2.5. Matrix effects

In terms of matrix effects, concentrations of samples containing 20, 75 and 150 ng/mL CVB-D were found to be 104.4%, 102.2% and 106.2% of the nominal concentrations, respectively. This indicates that matrix effects are not an issue using the current method.

3.2.6. Stability

In terms of stability, the results shown in Table 2 show that CVB-D is stable in human plasma and in processed samples under the conditions described.
4. Application

The present method was successfully applied to a pharmacokinetic study involving twenty-two healthy Chinese male volunteers after oral administration of a single dose of 2 mg CVB-D tablets. The mean plasma concentration versus time profile of CVB-D is shown in Fig. 3 and corresponding pharmacokinetic parameters are as follows:

\[
C_{\text{max}}, 12.1 \pm 5.5 \text{ ng/L}; T_{\text{max}}, 10 \pm 7 \text{ h}; \ t_{1/2}, 39 \pm 14 \text{ h}; \ AUC_{0-120}, 4398 \pm 1656 \text{ ng·h/L}; \ AUC_{0-\infty}, 5292 \pm 2034 \text{ ng·h/L}.
\]

<table>
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<tr>
<th>Nominal concentration (ng/L)</th>
<th>Intra-day (n=5)</th>
<th>Inter-day (n=15)</th>
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<tr>
<td>Determined mean concentration (ng/L)</td>
<td>RSD (%)</td>
<td>Mean accuracy (%)</td>
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<td>20</td>
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5. Conclusions

A novel LC–APCI–MS/MS method for the determination of CVB-D in human plasma has been developed and validated. The main advantages of the method include simple sample preparation by solid phase extraction and high sensitivity. This method is suitable for clinical pharmacokinetic studies as shown by its successful application in a pharmacokinetic study of CVB-D 2 mg tablets in healthy Chinese male volunteers.

References