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Short communication

Simultaneous determination of a novel anxiolytic agent buagafuran and one metabolite in human plasma by ultra-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A robust and validated ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method has been developed for the simultaneous determination of buagafuran and one metabolite (M1) in human plasma. The two analytes were extracted from plasma samples using tert-butyl methyl ether after addition of the internal standard and chromatographed on an Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm) thermostatted at 35 °C with methanol–water (75:25, v/v) as the mobile phase at an isocratic flow rate of 0.4 mL/min. The detection was performed on an API 5500 mass spectrometer coupled with electrospray ionization (ESI) source in positive mode. The multiple reactions monitoring (MRM) transitions of *m/z* 245.2→109.1 and *m/z* 279.1→243.1 were used to quantify buagafuran and M1, respectively. The assay was validated over the concentration range of 0.5–200 ng/mL for the two analytes. Precision and accuracy are in accordance with the generally accepted criteria for bioanalytical methods. The extraction recovery and the matrix effect were investigated. This method was successfully applied to support a clinical study where multiple oral doses were administrated to healthy Chinese volunteers to investigate the pharmacokinetics, safety and tolerability of buagafuran.

1. Introduction

Buagafuran (4-butyl-α-agarofuran, previously named as AF-5) is a synthetic derivative of agarofuran that possesses pharmacological activity on the central nervous system [1,2]. Compared to diazepam and buspirone, the significant antianxiety activity of buagafuran was more effective and less toxic, which may be attributed to its effects on central monoamine neurotransmitters [3].

During the preclinical study, the absorption of buagafuran was extremely poor with an absolute bioavailability below 9.5% [4], mainly due to the combination reaction of the intestine P-glycoprotein and the liver cytochrome P450 3A (CYP3A) enzyme [5,6]. The influence of buagafuran on the CYP enzymes in rats was investigated and the results showed that buagafuran significantly increased the activity and protein levels of CYP1A2 and CYP2E1 and had no effect on the CYP 3A [7]. In addition, the metabolism of buagafuran *in vitro* was studied with liver microsomes from rats. Five metabolites were isolated and structurally identified by means of MS and NMR in previous report [8] and these compounds were synthesized and purified again recently. It is necessary to know about the exposure of the main metabolites considering their safety in human based on the FDA guidelines for industry safety testing of drug metabolites [9], which also helps to assess the mass balance of the drug. However, only M1 gives relatively high response in human plasma after oral administration of buagafuran and no responses or very low responses were observed for other metabolites in MRM channels.

Up to now, only two researches reported the determination of buagafuran itself in plasma using GC–MS [10] and HPLC–MS/MS method, respectively [11]. Based on the previous report [11], a rapid and selective UPLC–MS/MS method was developed to determine the buagafuran and M1 simultaneously with buagafuran-*d*4 as the internal standard (IS) in the present study. The determination method was validated over the concentration range of 0.5–200 ng/mL for the two analytes. Precision and accuracy are in accordance with the generally accepted criteria for bioanalytical methods. The extraction recovery and the matrix effect were investigated. This method was successfully applied to support a clinical study where multiple oral doses were administrated to healthy Chinese volunteers to investigate the pharmacokinetics, safety and tolerability of buagafuran.

2. Experiment

2.1. Chemical and reagents

Buagafuran, M1 and buagafuran-*d*4 (shown in Fig. 1) were synthesized at Laboratory of Chemical Synthesis (Chinese Academy of

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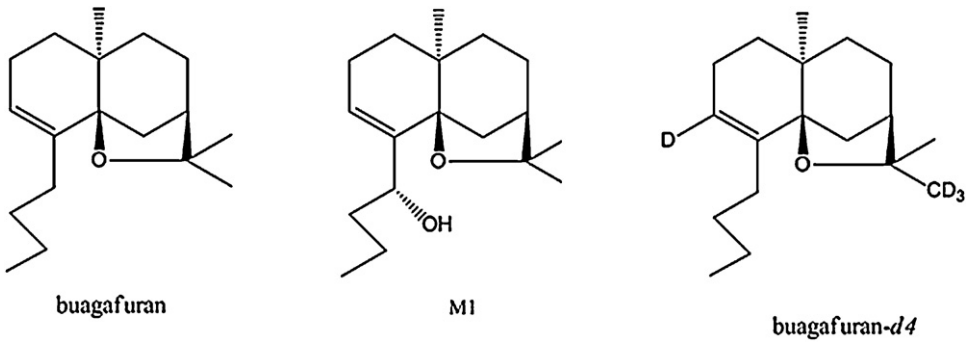

Fig. 1. Chemical structures of buagafuran, M1 and buagafuran-*d*4.

Medical Sciences) with purities of 99.4%, 97.8% and 98.0%, respectively. Methanol (HPLC grade) was purchased from Honeywell Burdick & Jackson (Muskegon, ML, USA) and tert-butyl methyl ether (HPLC grade) was from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade water was obtained using a Milli Q system (Millipore, Bedford, USA). Drug-free human plasma was supplied by Peking Union Medical College Hospital blood bank with K_3EDTA as the anticoagulant.

2.2. UPLC conditions and MS conditions

Acquity UPLC system (Waters Corp., Milford, MA, USA) consists of a binary pump, a seal wash pump, a solvent degasser, an automatic sample manager, and a thermostatted column compartment. Samples were automatically injected with a 10-μL syringe, and the analytes were separated on an Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm; Waters Corp., Milford, MA, USA) thermostatted at 35 °C. The mobile phase consisted of methanol–water (75:25, v/v). The samples were delivered at a flow rate of 0.4 mL/min and the injection volume was 3 μL.

A mass spectrometric analysis was performed using an API 5500 triple quadruple instrument from Applied Biosystems Sciex (Toronto, Canada) equipped with an electro-spray ionization (ESI) source operating in positive mode. Detection was achieved in multiple reactions monitoring (MRM) mode under unit mass resolution (0.7 amu @ FWHH) in both the Q1 and Q3 mass analyzers, and the dwell time set to 200 ms for each MRM transition. The MRM transitions were *m/z* 245.2→109.1, *m/z* 279.1→243.1 and *m/z* 249.2→113.1 for buagafuran, M1 and IS, respectively. The mass spectrometric conditions were optimized as follows: source temperature, 400 °C; curtain gas, 40 units; nebulizer gas, 70 units; turbo gas, 70 units; collision gas, 7 units; ionspray voltage, 4000 V; declustering potential, 50 V; collision energy, 27 eV for buagafuran and IS, 10 eV for M1. Data acquisition and processing were performed using Analyst software (version 1.5.1).

2.3. Preparation of calibration standards (CS) and quality controls (QC)

Two separate combined stock solutions were prepared at concentrations of 1 mg/mL for buagafuran and M1 in methanol, which were used for the preparation of CS and QC samples, respectively. They were further diluted with methanol to yield working solutions at a concentration of 10 μg/mL for the two analytes.

CS and QC samples in plasma were prepared by diluting the corresponding working solutions with drug-free human plasma. The final concentrations in plasma were 0.5, 1, 5, 10, 20, 50, 100 and 200 ng/mL, respectively. The concentrations of lower limit of quantitation (LLOQ) samples and three pools of QC samples were 0.5, 1.5, 20 and 150 ng/mL, respectively. The working solution of internal standard (IS) was prepared in methanol at a concentration of 150 ng/mL.

All the stock solutions, working solutions, calibration standards and quality control samples were stored at −30 °C and were brought to room temperature before analysis.

2.4. Samples preparation

CS, QC and clinical samples were purified by simple liquid–liquid extraction method. A volume of 50 μL IS working solution was added to 200 μL of plasma sample in a 2 mL eppendorf tube. After vortexed for 30 s on a shaker, 1.2 mL of tert-butyl methyl ether was added. The mixture was vortexed for 2 min and then centrifuged at 13,000 rpm for 5 min. The clear supernatants were collected and evaporated to dryness under a stream of nitrogen at room temperature. The residues were dissolved in 100 μL of methanol–water (70:30, v/v) and vortexed for 1 min. Finally, 3 μL of the sample was injected for UPLC–MS/MS analysis.

2.5. Method validation

The method was validated for selectivity, sensitivity, linearity, accuracy and precision, stability of the analytes at various test conditions, recovery and matrix effect according to the FDA guidelines for biological method validation [12].

The selectivity of the assay was investigated by analyzing six lots of analyte-free plasma. Endogenous interferences were observed in different lots of plasma sample, and the "cross-talk" between MRM transitions was evaluated by analyzing the different blank plasma sample, which was only spiked with buagafuran, M1 at a concentration of upper limit of quantitation (200 ng/mL) or IS of 150 ng/mL.

The sensitivity of the method was evaluated using LLOQ by analyzing five replicates in the same run. A precision of ±20% and an accuracy of 80–120% were allowed for the LLOQ [12].

The calibration curves were constructed using 8 non-zero standards ranging from 0.5 to 200 ng/mL. The linearity of the relationship between the peak area ratio and the concentration was demonstrated by the correlation coefficient (R) obtained from the linear regression. The relative standard deviations were calculated for all slopes of calibration curves. Five replicates of QC samples at low, medium and high concentration were analyzed to evaluate the intra- and inter-day precision and accuracy. The relative standard deviation (RSD%) and relative error (RE%) were calculated and could not exceed ±15%.

In order to evaluate the short-term stability, three levels of QC samples were extracted and determined after being placed at room temperature (25 °C) for 8 h. The auto-sampler stability was done