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# Quantitative determination of buagafuran in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive and selective high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was developed for the determination of buagafuran in human plasma. The analyte was extracted from plasma samples with hexane after addition of isotopic internal standard and chromatographed on a RP-$C_8$ column. The mobile phase consisted of methanol–water (90:10, v/v) and the flow rate was 0.2 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer in multiple reactions monitoring (MRM) mode using positive electrospray ionization (ESI). The method was validated over the concentration range of 0.5–200 ng/mL. Inter- and intra-day precision (RSD%) were all within 15% and the accuracy (RE%) was equal or lower than 9.5%. The lower limit of quantitation (LLOQ) was 0.5 ng/mL. The extraction recovery was on average 38.1% and the detection was not affected by the matrix. The method was successfully applied to the pharmacokinetic study of buagafuran in healthy Chinese volunteers.



## 1. Introduction

Anxiety disorders have become one of the most common psychiatric difficulties throughout the life course [1]. It was found that anxiety disorders had a complicated underlying progress in a number of forms, causing considerable disconsiderable distress to individuals, families and society [2]. Pharmacotherapy remains the first-line treatment although the therapeutic approaches are diverse [3].

Buagafuran (4-butyl-a-agarofuran, $C_{18}H_{30}O$, MW 262.4, previously named as AF5) is a synthetic derivative of agarofuran, which exhibits significant anti-anxiety activity in several animal models, with higher potency and lower toxicity compared with diazepam and buspirone [4]. The possible anti-anxiety mechanism is related to the modulation of central monoamine neurotransmitters [5]. Buagafuran was approved for treatment of anxiety disorders by China State Food and Drug Administration (SFDA) in 2007 and is currently being evaluated in phase I trials.

To date, few references are available about the determination of buagafuran except a GC–MS method [6], which showed the lower limit quantitation was 2.5 ng/mL in dog plasma. Because the concentration of buagafuran in human plasma is low, a more sensitive method is required for clinical study. In this study, a LC–MS/MS method was established and validated according to FDA guideline [7]. The method proved to be more sensitive and selective, and was successfully applied to the pharmacokinetic studies of buagafuran in Chinese healthy volunteers.

## 2. Materials and methods

### 2.1. Chemical and reagents

Buagafuran (Fig. 1) and its internal standard buagafuran-*d*4 were synthesized at Laboratory of Chemical Synthesis (Chinese Academy of Medical Sciences). Methanol (HPLC grade) was purchased from Honeywell Burdick & Jackson (Muskegon, ML, USA) and hexane (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade water was obtained using a Milli Q system. (Millipore, Bedford, USA). Drug-free human plasma used in the research was supplied by Peking Union Medical College Hospital blood bank.

### 2.2. HPLC conditions

The chromatographic separation was carried out on a Waters Alliance 2690 liquid chromatograph system (Waters, USA). A reversed-phase Symmetry C8 column (3.5 μm, 50 × 2.0 mm i.d.; Waters Co., Ltd., USA) was used for all the separations. The mobile phase consisted of methanol:$H_2O$ (90:10, v/v). The samples were delivered at a flow rate of 0.2 mL/min and the injection volume was 10 μL.

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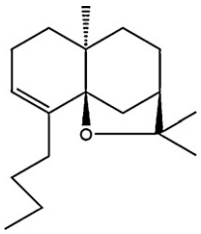

**Fig. 1.** Chemical structure of buagafuran.

### 2.3. MS conditions

Mass spectrometric analysis was performed using API 3000 triple quadruple mass spectrometer from Applied Biosystems Sciex (Toronto, Canada) equipped with electro-spray ionization (ESI) source in the positive mode. The detection was operated in the multiple reaction monitoring (MRM) mode with a dwell time of 200 ms for each transition. The MRM transitions of buagafuran and its internal standard (IS) were 263.1 → 245.2 and 267.2 → 249.2, respectively. The mass spectrometric condition was optimized as follows: source temperature, 150 °C; curtain gas, 9 units; nebulizer gas, 6 units; auxillary gas (7000 units); collision gas, 5 units; ionspray voltage, 5000 V; declustering potential, 15 V; collision energy, 12 eV. Ultrahigh-purity nitrogen gas was used as the collision gas. Data acquisition and processing were performed using Analyst software (version 1.4).

### 2.4. Preparation of standard solutions, calibration standards and quality controls

Stock solutions of buagafuran were prepared in duplicate in methanol at concentration of 1 mg/mL, one for calibration curve samples and the other for quality control (QC) samples. The stock solution of internal standard (IS) was prepared as above to obtain the concentration of 1 mg/mL. Fresh working solutions were prepared by diluting the stock solution to the concentration of 10 μg/mL for buagafuran and 150 ng/mL for IS. Calibration standards were prepared by diluting the corresponding working solutions with drug-free human plasma. The final concentrations of buagafuran in plasma were 0.5, 1, 2, 10, 20, 50, 100, 250 ng/mL. LLOQ samples and three pools of quality control (QC) samples were prepared by spiking blank plasma with buagafuran at the concentrations of 0.5, 1.5, 20 and 200 ng/mL, respectively. All the stock solutions, working solutions, calibration standards and quality controls were stored at −30 °C and were brought to room temperature before analysis.

### 2.5. Samples preparation

A volume of 200 μL plasma was transferred into a 10 mL test tube followed by addition of 50 μL of IS working solution (150ng/mL). After the glass tube was vortexed for 30 s, 1.2 ml of hexane was added. The mixture was vortexed for 2 min and then centrifuged at 3000 × *g* for 5 min. The supernatant was collected and evaporated to dryness under a stream of nitrogen at room temperature. The residues were dissolved in 100 μL of methanol:$H_2O$ (8:2, v/v) and vortexed for 1 min. Finally, 10 μL of the sample was injected for LC–MS/MS analysis.

### 2.6. Method validation

Validation of this method was performed in compliance with FDA guidelines for biological method validation [7], including selectivity, calibration curve performance, accuracy and precision, LLOQ, stability of the analytes at various test conditions, recovery and matrix effect.

Selectivity was assessed by comparing the chromatograms of drug-free human plasma with the corresponding spiked plasma for the test of endogenous interferences.

The calibration curves were constructed using 8 non-zero standards ranging from 0.5 to 250 ng/ml. The linearity of the relationship between peak area ratio and concentration was demonstrated by the correlation coefficient ($R$) obtained for the linear regression. The relative standard deviation was calculated for all slopes of calibration curves. The intra- and inter-day assays of the method were evaluated by assessing 5 replicates QC samples at low, medium and high concentration. Relative standard deviation (RSD%) and relative error (RE%) were calculated.

The stability of buagafuran stock solution was calculated by comparing the peak area of stock solution after being stored for a period of time with that of solution freshly prepared at the same concentration. 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 24 h. The post-extraction stability was done by comparing the concentration of extracted QC samples after being kept at autosampler for 24 h with the nominal concentration. Freeze-thaw stability was determined by comparing the value of QC samples after 3 freeze/thaw cycles with the nominal value (from −30 °C to 25 °C). Long-term storage stability was acquired by assessing QC samples stored at –30 °C for 108 days.

The extraction recovery of buagafuran was calculated by direct comparison of the peak area of extracted three levels QCs to that of the analytes spiked to the blank sample post-extraction at the same concentration.

To determine the matrix effect, six different blank plasma samples were utilized to prepare the QC samples at 3 levels concentrations and used for assessing the lot-to-lot matrix effect. The matrix effect was evaluated with the average peak areas in spiked samples post-extraction to that of corresponding working solutions at same concentration.

### 2.7. Data treatment

Data acquisition was performed using Analyst software (version 1.4). Descriptive statistics (mean, SD, RSD%, RE%) were calculated using Watson LIMS (version 7.3). Peak area ratios of the analytes to internal standard were utilized to construct calibration curves and the linear regression between plasma concentration and peak area ratio was weighted by $1/x^2$. Concentrations of QCs and unknown samples were calculated by interpolation from the calibration curves.

## 3. Results and discussion

### 3.1. LC–MS/MS method optimization

In this study, a LC–MS/MS method for the detection of buagafuran in human plasma was investigated. The analytes were introduced into mass spectrometer using turbo ionspray interface, and the highest signal intensity for the analytes was found when using the ESI source in a positive ionization mode. MRM scan mode was selected to ensure high specificity of this method.

In general, the dehydrated fragment ion is easy to be generated in the mode of in-source collision-induced dissociation, resulting in a high background noise level in chromatography and decreasing the sensitivity and specificity of the method [8]. However, a main fragment ion corresponding to loss of water was finally selected for the MRM detection of buagafuran, because it was found that the peak from the loss of water, i.e. $[M+H-H_2O]^+$ ($m/z$ 245) is the

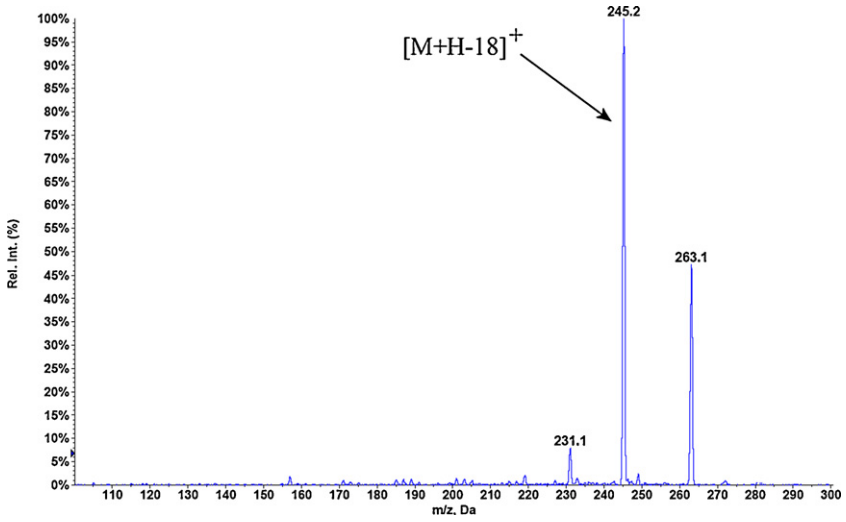


**Fig. 2.** Product ion spectrum of buagafuran.

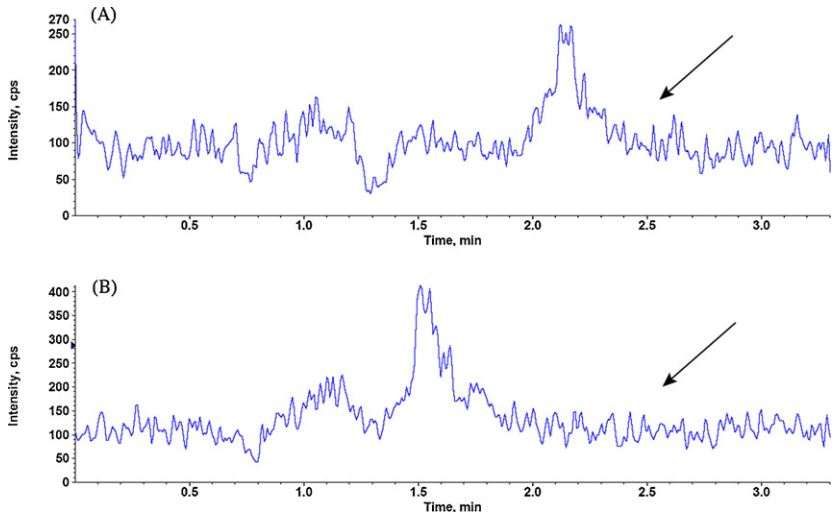


**Fig. 3.** Representative MRM chromatograms of buagafuran (A) and IS (B) from a blank plasma sample without any analyte and internal standard added.

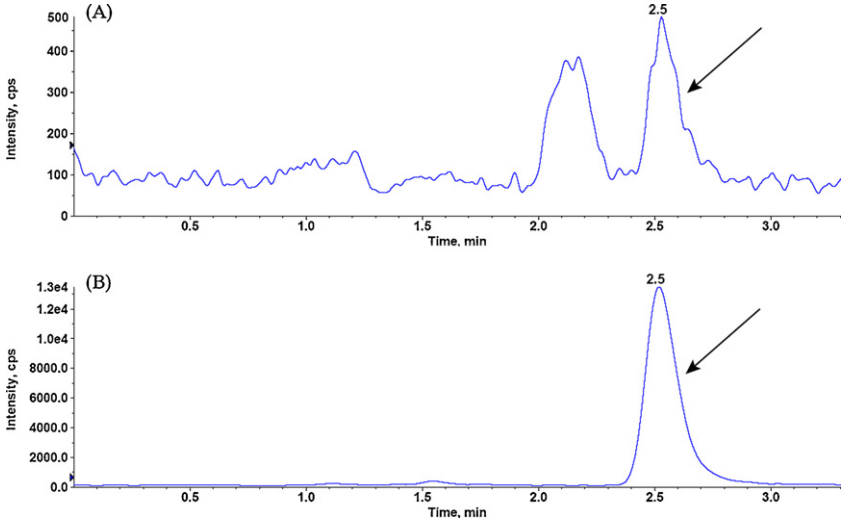


**Fig. 4.** Representative MRM chromatograms of buagafuran (A) and IS (B) from the LLOQ sample (0.5 ng/mL).

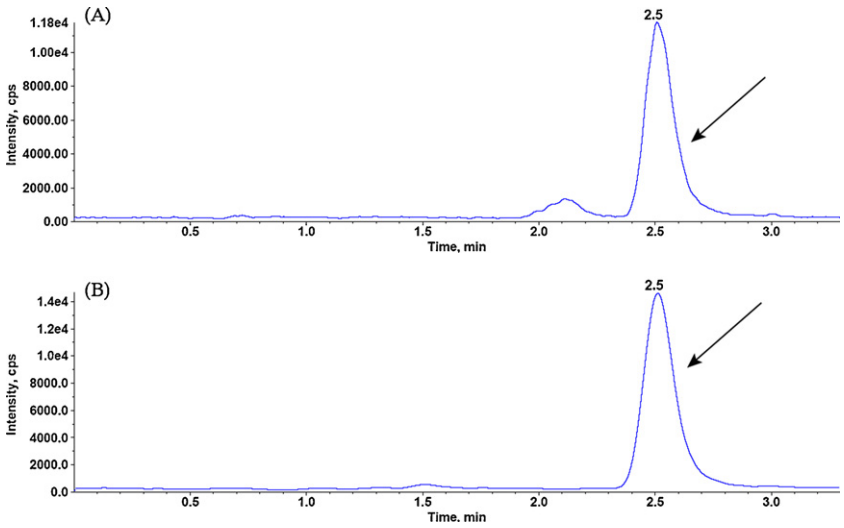


**Fig. 5.** Representative MRM chromatograms of buagafuran (A) and IS (B) from a QC sample at 20 ng/mL.

most abundant in the product ion spectrum of buagafuran (Fig. 2). Furthermore, blank samples of each matrix were examined without any interference peak in channel (Fig. 3) and high signal to noise ratio (S/N = 6) was observed in chromatogram (Fig. 4).

In order to obtain the most stable and the highest signal response of the MRM transition, the mass spectrometric parameters were optimized, including ionspray voltage, source temperature, nebulizer gas, collision gas and collision energy.

Furthermore, good separation from interference of matrix was obtained by optimizing the mobile phase system, including the kinds and the different ratios of the solvents. In our extensive preliminary experiments, methanol, acetonitrile, formic acid and buffer solution (ammonium acetate with different pH valve) had been investigated. Additional acid or buffer solution could not help to increase the S/N ratio and there were no difference between methanol and acetonitrile to separate the analyte from the interference. Best results in terms of peak shape, retention time and separation from interference were obtained using the simple isocratic gradients of methanol/water (90:10, v/v). The isotope internal standard buagafuran-*d*4 was applied to get more accuracy because of the similar chromatographic behavior and ionization efficiency to the analyte.

### 3.2. Method validation

#### 3.2.1. Selectivity

Representative MRM chromatograms of blank plasma samples (Fig. 3) from 6 different lots, plasma sample spiked with drugs (Fig. 5) and a real human plasma sample (Fig. 6) were obtained under the selected analytical conditions. The result showed the absence of any interference at the retention time of the analyte and IS.

#### 3.2.2. Sensitivity

The chromatogram of LLOQ sample was used to evaluate the sensitivity of the method. The LLOQ was set at 0.5 ng/mL and a typical chromatography of plasma sample containing 0.5 ng/mL buagafuran is shown in Fig. 4. Five replicates of plasma samples at LLOQ level were investigated and the results met the acceptance criteria with accuracy of 16.2% (RSD%) and precision of 16% (RE%).

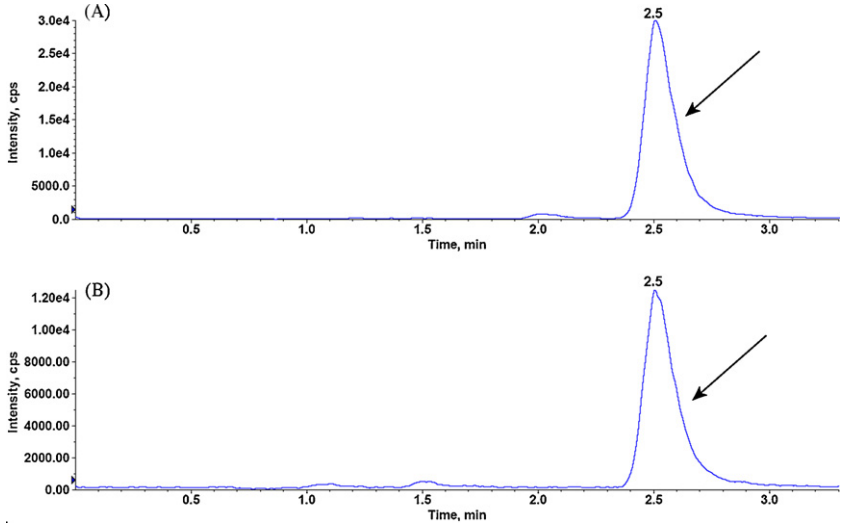


**Fig. 6.** Representative MRM chromatograms of buagafuran (A) and IS (B) from a real human plasma sample collected at 2 h after oral administration of 120 mg buagafuran (64.7 ng/mL).

**Table 1**
Back-calculated calibration standards of buagafuran ($n = 3$).

| Conc. (ng/mL) | 0.5000 | 1.000 | 2.000 | 10.00 | 20.00 | 50.00 | 100.0 | 250.0 | *R*-squared |
|---|---|---|---|---|---|---|---|---|---|
| Mean | 0.5006 | 0.9394 | 2.212 | 9.758 | 22.43 | 49.04 | 95.21 | 234.6 | 0.9933 |
| SD | 0.01 | 0.05 | 0.05 | 0.09 | 0.49 | 2.20 | 2.82 | 6.27 | 0.0021 |
| RSD% | 2.2 | 5.1 | 2.1 | 0.9 | 2.2 | 4.5 | 3.0 | 2.7 | 0.2 |
| RE% | 0.1 | −6.1 | 10.6 | −2.4 | 12.2 | −1.9 | −4.8 | −6.2 | / |

**Table 2**
Intra- and inter-day accuracy and precision of quality control samples.

| | Intra-day (ng/mL) | | | Intra-day (ng/mL) | | |
|---|---|---|---|---|---|---|
| | Low (1.500) | Medium (20.00) | High (200.0) | Low (1.500) | Medium (20.00) | High (200.0) |
| Mean | 1.486 | 19.16 | 195.2 | 1.416 | 18.83 | 189.5 |
| SD | 0.14 | 1.82 | 5.22 | 0.12 | 1.06 | 5.91 |
| Precision (RSD%) | 9.4 | 9.5 | 2.7 | 8.8 | 5.6 | 3.1 |
| Accuracy (RE%) | −0.9 | −4.2 | −2.4 | −5.6 | −5.9 | −5.3 |
| n | 5 | 5 | 5 | 15 | 15 | 15 |

**Table 3**
Stability results of QC samples at different conditions ($n = 5$).

| Stability tests | Theoretical conc. (ng/mL) | Found conc. (ng/mL) | SD | Precision (RSD%) | Accuracy (RE%) |
|---|---|---|---|---|---|
| Stock solution (−30 °C for 5 months) | 96,300[a] | 83,700 | 1970 | 2.3 | −13.1 |
| Short term (25 °C for 24 h) | 1.500 | 1.361 | 0.14 | 10.5 | −9.3 |
| | 20.00 | 19.10 | 0.80 | 4.2 | −4.5 |
| | 200.0 | 179.3 | 7.19 | 4.0 | −10.4 |
| Post-extraction (15 °C for 24 h) | 1.500 | 1.288 | 0.15 | 12.0 | −14.2 |
| | 20.00 | 18.76 | 0.28 | 1.5 | −6.2 |
| | 200.0 | 182.2 | 1.97 | 1.1 | −8.9 |
| Freeze thaw (from −30 °C to 25 °C, 3 cycles) | 1.500 | 1.294 | 0.11 | 8.2 | −13.7 |
| | 20.00 | 18.55 | 0.51 | 2.7 | −7.3 |
| | 200.0 | 190.0 | 4.69 | 2.4 | −5.0 |
| Long term (−30 °C for 108 days) | 1.500 | 1.565 | 0.04 | 2.7 | 4.3 |
| | 20.00 | 20.38 | 0.72 | 3.6 | 1.9 |
| | 200.0 | 212.5 | 7.43 | 3.5 | 6.3 |

[a] Peak area of fresh stock solution.

### 3.2.3. Linearity, precision and accuracy

The calibration curves were validated over the concentration range of 0.5–250 ng/mL in human plasma. Correlation coefficients of all the curves were greater than 0.99 and the deviations of the back-calculated concentrations from their nominal values were within ±15%. The precision and accuracy for each standard concentration are shown in Table 1 ($n = 3$).

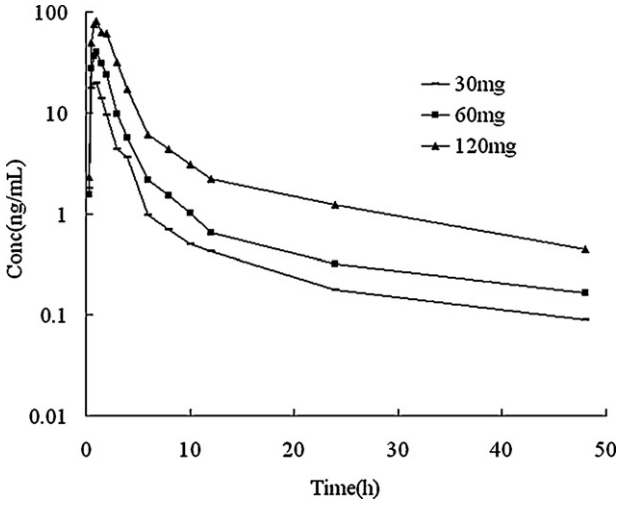


**Fig. 7.** Mean plasma concentration–time curves of buagafuran after single oral administration of 30 mg, 60 mg and 120 mg buagafuran ($n = 12$).

The intra- and inter-day precision and accuracy of the method were determined from the analysis of 5 QC samples at three different concentrations and the results are summarized in Table 2 ($n = 5$). It was demonstrated that the method was reliable and reproducible since RSD% was below 15% and RE% was equal or lower than 9.5% for all the investigated concentrations.

### 3.2.4. Stability

Buagafuran stock solution was stable at −30 °C for 5 months. The stability of the analyte in human plasma was investigated under a variety of storage and processing conditions and it was found to be stable under conditions as follows: at room temperature (25 °C) for 24 h; at 15 °C for 24 h post-extraction; after 3 cycles freeze/thaw (from −30 °C to 25 °C); at −30 °C for 108 days (Table 3, $n = 5$).

### 3.2.5. Recovery and matrix effect

A liquid/liquid method was introduced to the extraction of the analyte. The extraction recoveries were 33.7% ± 2.5%, 40.0% ± 1.8% and 40.4% ± 2.3% for low, medium and high concentration of QC samples ($n = 5$), respectively.

No apparent matrix effect was found to affect the determination of buagafuran. The mean value was 94.2% (ranged from 87.1% to 106.4%, RSD% was 8.6%) for 3 levels of QC samples ($n = 6$).

### 3.3. Clinical application

This HPLC/MS/MS method was used to evaluate the pharmacokinetic study of buagafuran in human plasma. This was a Phase I,

open-label, randomized, cross-over design and dose-ranging study to assess the pharmacokinetics of buagafuran in healthy Chinese subjects. Twelve healthy male Chinese volunteers were enrolled and received a single oral dose of 30, 60, 120 mg buagafuran. Plasma samples were colleted at specific time points and assayed by the validated method. Mean plasma concentration–time curves of buagafuran after single oral administration of 30 mg, 60 mg and 120 mg buagafuran is shown in Fig. 7.

## 4. Conclusion

A rapid, sensitive and selective HPLC–MS–MS method has been developed and validated for the determination of buagafuran in human plasma. The method, using a simple liquid/liquid extraction of buagafuran and IS from plasma, is sensitive, specific and accurate. The present method was successfully applied to access pharmacokinetic studies of buagafuran in healthy Chinese volunteers.

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## References

[1] R.C. Kessler, P. Berglund, O. Demler, R. Jin, K.R. Merikangas, E.E. Walters, Arch. Gen. Psychiatry 62 (2005) 593–602.
[2] A. Garakani, S.J. Mathew, D.S. Charney, Mt. Sinai J. Med. 73 (2006) 941–949.
[3] J.R. Davidson, J. Clin. Psychiatry 70 (Suppl. 2) (2009) 25–31.
[4] Q. Liu, D.H. Wang, C. Li, D. Lu, W.J. Wang, J.Y. Guo, Chin. J. Med. Chem. 13 (2003) 125–130.
[5] Y. Zhang, W.J. Wang, J.J. Zhang, Eur. J. Pharmacol. 504 (2004) 39–44.
[6] J.P. Hu, H. Chen, Y. Li, J. Pharm. Biomed. Anal. 47 (2008) 383–387.
[7] Food and Drug Administration (FDA), Guidance for Industry in Bioanalytical Method Validation, Rockville, MD, USA, 2001.
[8] M.M. Kushnir, A.L. Rockwood, G.J. Nelson, B. Yue, F.M. Urry, Clin. Biochem. 38 (2005) 319–327.