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Development of a gas chromatography–time-of-flight mass spectrometry method for the determination of buagafuran, a promising antianxiety drug in dog blood

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Abstract

A sensitive gas chromatography–time-of-flight mass spectrometry (GC–ToFMS) method has been developed and validated for the determination of buagafuran, a new anxiolytic drug derived from α-agarofuran. Buagafuran and internal standard (buagafuran-d_4) were isolated from plasma by liquid–liquid extraction. The separation was achieved on HP-1 capillary column (25 m × 0.2 mm × 0.11 μm). Buagafuran (*m*/*z* 262.22) and buagafuran-d_4 (*m*/*z* 266.25) were eluted at 7.6 min and no endogenous materials interfered with the measurement. The calibration curves of buagafuran were linear over the range of 2.5–160 ng/ml in dog plasma. The lower limit of quantification (LLoQ) was 2.5 ng/ml in plasma. The within-day and between-day precisions were less than 15%. The method was used to determine the plasma concentration–time profile of buagafuran after oral doses of 8, 16, 32 mg/kg in dogs.
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Keywords: Buagafuran; Gas chromatography–time-of-flight mass spectrometry (GC–ToFMS); Pharmacokinetic; Dog; Anxiety disorders

1. Introduction

Anxiety disorders are the most common type of psychiatric disorders, with an incidence of 18% and a life prevalence of 29% in the population [1,2]. It was found that anxiety disorders had a complicated underlying process and presented in a number of forms, causing considerable distress to individuals, families and society [3]. Pharmacotherapy of anxiety disorders remains the most widespread and efficacious treatment, and the development of new antianxiety drugs is a very active field.

Sesquiterpinoids, e.g. α-agarofuran from the Chinese traditional medicine Gharu-wood (*Aquillaria agallocha* Roxb.) have attracted much interest due to their structural diversities and broad spectrum of biological activities, including sedative, hypnotic, appetite suppressive, antiemetic, and antibacterial activities. Buagafuran (4-butyl-α-agarofuran, Fig. 1, previously named as AF-5) is a synthetic derivative of agarofuran [4–6]. Previous studies had shown that buagafuran had significant antianxiety activity in several animal models, with a higher potency and a lower toxicity compared with diazepam and buspirone. The mechanism was related to the modulation of central monoamine neurotransmitters [7,8]. The aim of this study was to investigate the pharmacokinetics of buagafuran in beagle dogs' plasma samples. A sensitive, accurate and reproducible method was necessary for this purpose. We selected gas chromatography–time-of-flight mass spectrometric (GC–ToFMS) technique using stable isotope tracer since this technique was successfully used in the recent years for various bioanalytical and other purposes [9,10].

2. Experimental

2.1. Chemicals and reagents

Buagafuran (purity >99.8%) and internal standard (IS, buagafuran-d_4) were synthesized at Laboratory of Chemical Synthesis (Chinese Academy of Medical Sciences). Hexane and other reagents were obtained from Sinopharm Chemical Reagent Beijing Co. Ltd. All reagents were of analytical grade.

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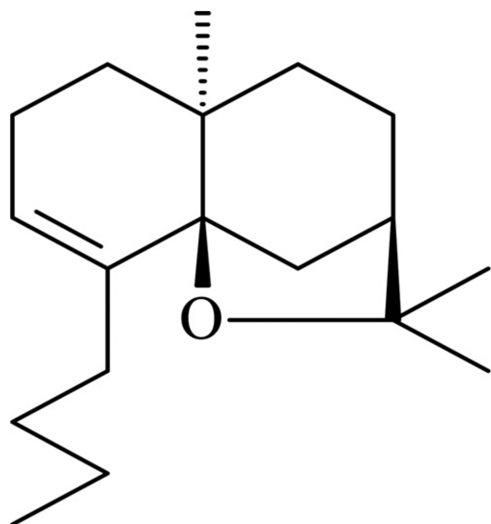

Fig. 1. Chemical structure of buagafuran.

Drug-free plasma was obtained from untreated beagle dogs weighing 6–8 kg purchased from Beijing Marshall Inc. (Beijing, China).

2.2. Preparation of stocks, calibration standards and quality control samples

Buagafuran was dissolved in hexane (1 mg/ml) as stock solution. Working standards were prepared freshly by diluting the stock solution with hexane.

Calibrations standards were prepared by adding different concentrations of the working standards to drug-free plasma. The final concentrations of buagafuran were 2.5, 5, 10, 20, 40, 80, 160 ng/ml, and IS was 20 ng/ml, respectively. A 1 ml buagafuran sample was spiked with 4 ml hexane and the mixtures were vortexed for 1 min. After centrifugation at $3000 \times g$ for 5 min, the upper layer was transferred to another tube. The blood samples were extracted twice and the upper organic portions were combined and evaporated to dryness under nitrogen at 40 °C. The residue was dissolved in 20 μl of hexane, and 1 μl of aliquot was injected into the GC–MS system.

High-, mid- and low-level quality control samples contained 80, 20 and 5 ng/ml each of buagafuran analytes. The samples were prepared in the same manner as preparation of the calibrator samples.

2.3. GC–ToFMS chromatography

GC–ToFMS analysis was performed on an Agilent 6890 GC system (Agilent Corp, USA) equipped with a time-of-flight mass spectrometry (MicroMass, UK). A HP-1 capillary column (25 m × 0.2 mm × 0.11 μm) was employed with helium as carrier gas. The oven temperature program was set as follow: initial temperature, 70 °C for 1 min; gradient of 30 °C/min until 160 °C; gradient of 10 °C/min until 210 °C; gradient of 20 °C/min until 250 °C; hold time, 2 min. The injector temperature was 240 °C and 1 μl was injected in the splitless mode. The TOFMS was operated using electron impact (EI) ionization mode and the electron collision energy was 70 eV. An ion source temperature of 200 °C, an interface temperature of 200 °C, and detector voltage of 2.7 kV were used. The full scan range of the sample for the qualitative analysis was from *m/z* 50 to *m/z* 500 and then the quantification was based on the extracted ion chromatograms from the full scans. The extracted ion traces *m/z* 262.2 (buagafuran) and *m/z* 266.2 (IS) were used for the quantification. The raw data acquired of buagafuran was analysed by MassLyns V4.0 software.

2.4. Bioanalytical method validation

2.4.1. Linearity, precision and accuracy

The linearity of the GC–ToFMS method for the determination of buagafuran was evaluated by a calibration curve (peaks' area of buagafuran/IS) in the range of 2.5–160 ng/ml. Least squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. The calibration curve requires a correlation coefficient (r^2) of 0.99 or better. To evaluate the precision, at least five QC samples of three different concentrations of buagafuran were processed and injected on a single day (intra-day) and at different days (inter-day). The variability of buagafuran determination was expressed as the coefficient of variation (% CV) which should be ≤15% at all the concentrations. Accuracy is expressed as % bias which should be within limits of ±15% at all concentrations of buagafuran.

2.4.2. Recovery

The extraction recovery of buagafuran was determined by calculating the ratio of the amount of extracted compound from drug-free blood spiked with known amounts of buagafuran to the amount of compound added at the same concentration to hexane. The recoveries at three QC concentration levels of buagafuran in plasma were examined at least five times.

2.4.3. Stability studies

The freeze–thaw stability of buagafuran in plasma samples was determined over three freeze–thaw cycles. In each freeze–thaw cycle, the samples were frozen and stored at −20 °C for 24 h, then thawed at ambient temperature. To evaluate long-term stability, the plasma samples were stored at −20 °C for 10 days. For the short-term stability, fresh plasma samples were kept at room temperature for 12 h before extraction.

2.5. Pharmacokinetic experiments in dogs

The Institute Animal Care and Welfare Committee approved all animal protocols. Male and female beagle dogs weighing 6–8 kg purchased from Beijing Marshall Inc. (Beijing, China) were housed in individual cages during the studies. Dogs were fasted 12 h before receiving buagafuran and fed 4 h after administration. The dosing solutions used for all animal studies were prepared by suspending the required amounts of buagafuran in 0.5% sodium carboxymethyl cellulose. The plasma pharmacokinetics of buagafuran was studied in male and female beagles (3 per sex) after a single-oral dose (8, 16, 32 mg/kg) and multiple

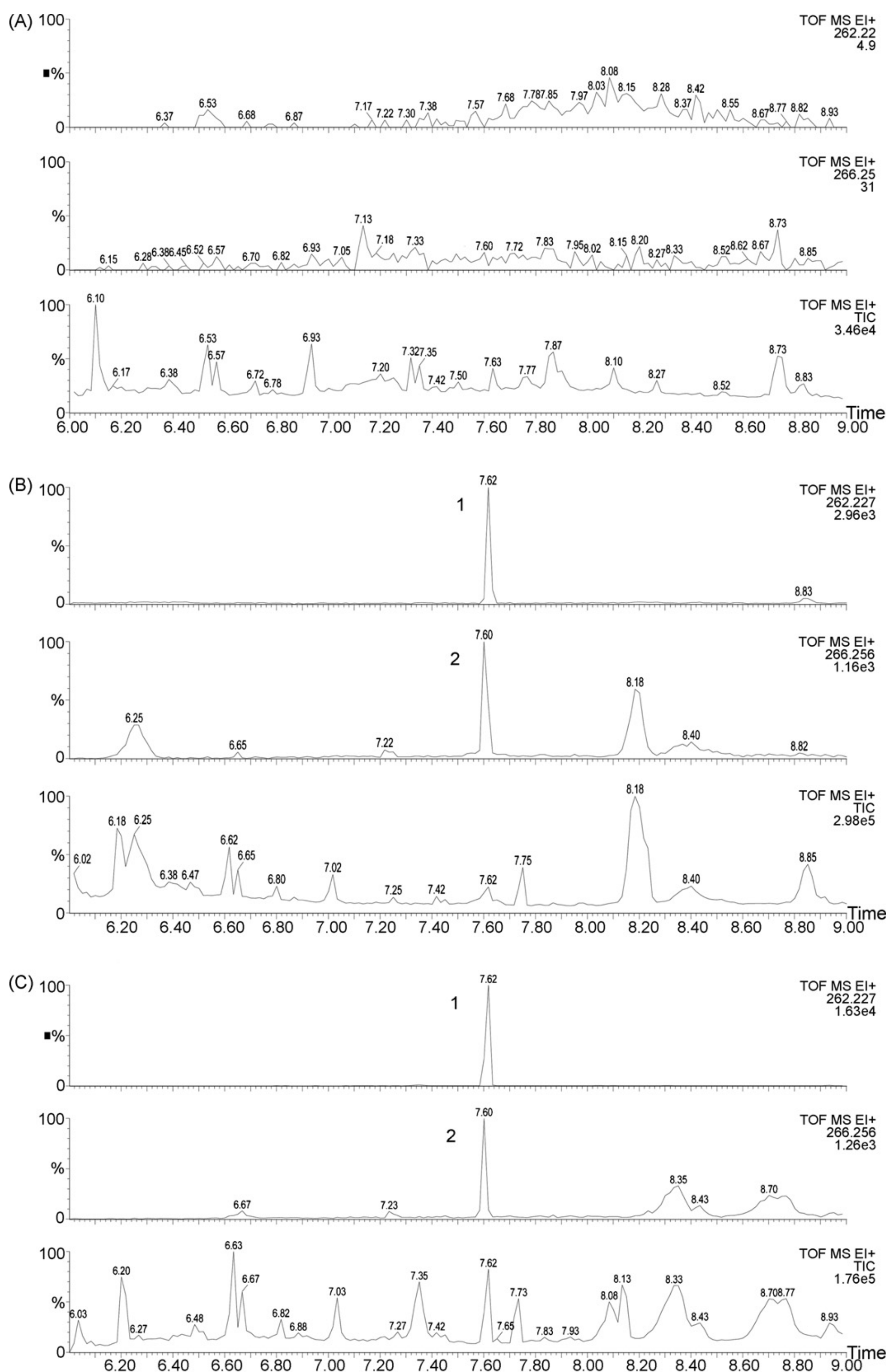

Fig. 2. Total ion chromatography (TIC), extracted ion chromatograms (EIC) of the blank plasma (A), the plasma spiked with buagafuran and buagafuran-d_4 (IS) (B), and dosed plasma (C). Peak 1 is buagafuran (m/z 262.2 Da) and Peak 2 is buagafuran-d_4 (m/z 266.2 Da).

doses (daily oral 16 mg/kg for 7 days). Blood samples were collected via the forearm vein into heparinized syringes at 0.08, 0.17, 0.33, 0.67, 1, 2, 3, 4, 6, 8, 12 and 24 h after oral dosing, centrifuged immediately and the resulting plasma prepared according to the procedure given for the calibrators. Pharmacokinetic analysis of buagafuran concentrations in plasma was performed using noncompartmental methods via the proprietary drug and statistics (DAS) computer software package (Version 2.0, Anhui Province Center for Drug Clinical Evaluation, China).

3. Results and discussion

3.1. Method development

The analytical method of α-agarofuran and its derivatives extracted from plants by gas chromatography–mass spectrometry was reported previously [11]. However, the lower limit of quantification (LLoQ) of method was 10 ng/ml and not sensitive enough to analyze buagafuran in dog plasma. In order to eliminate these obstacles, a liquid–liquid extraction step followed by gas chromatography–time-of-flight mass spectrometry method was established with high sensitivity (LLoQ 2.5 ng/ml). Hexane was found to be the most suitable solvent compared with diethyl ether, ethyl acetate, chloroform, dichloromethane, and petroleum ether. As internal standard buagafuran-d_4 was adopted to get high accuracy.

The chromatograms of blank plasma, buagafuran quality control (40 ng/ml) and dog plasma after an oral dosing of buagafuran (32 mg/kg) are shown in Fig. 2. The retention times of buagafuran and buagafuran-d_4 were 7.6 min. The extracted ion traces m/z 262.22 (buagafuran) and m/z 266.25 (buagafuran-d_4) were used for the quantification. No other endogenous compounds in dog plasma produced mass ions at m/z 262.22 and 266.25. The results showed that GC–ToFMS was suitable and reliable for the pharmacokinetics study of buagafuran in dog plasma.

3.2. Method validation

The quantification was achieved by the chromatographic peak area ratio of buagafuran to the calibration standards at the concentration range of 2.5–160 ng/ml. The standard curve was linear over the range of 2.5–160 ng/ml in dog plasma, and the mean values of regression equation were $Y = 0.1198X + 0.3$ ($r^2 = 0.9997$). The LLoQ of buagafuran was found to be 2.5 ng/ml which was 10 times of signal/noise ratio in dog plasma.

The intra- and inter-day variability of the assay for plasma is listed in Table 1. It demonstrated that the GC–ToFMS method for the determination of buagafuran was reliable and reproducible since both % CV and % bias were below 15% for all estimated concentrations of buagafuran.

The extraction recovery was determined for five replicates of dog plasma spiked with low, medium and high concentrations (5, 20 and 80 ng/ml) of buagafuran. The results are summarized in Table 2. The extraction recovery of buagafuran from the plasma was concentration independent in the concentration range evaluated and was considered to be acceptable.

3.3. Stability

The stability of buagafuran during the determination was assessed under a variety of conditions. Analysis of these samples consistently afforded values that were nearly identical to those of freshly prepared QC samples, thus confirming the overall stability of buagafuran in plasma under frozen storage, assay processing and freeze–thaw conditions.

3.4. Pharmacokinetic study of buagafuran in dogs

The validated assay was used to determine the plasma pharmacokinetic profiles of buagafuran in beagle dogs after a single-oral dose (8, 16, 32 mg/kg) and multiple doses (daily oral 16 mg/kg for 7 days). The mean plasma concentration versus time profiles of buagafuran after single-oral doses are shown in Fig. 3, and the mean pharmacokinetic parameters are summarized in Table 3. The plasma pharmacokinetic profile of buagafuran obtained following oral doses was characterized by multiple-peak curves. The fluctuations in the plasma concentration versus time profile had an impact on the apparent terminal

Table 2
Recovery of buagafuran in dog plasma

Matrix	Added (ng/ml)	n	Measured (ng/ml)	Recovery ± S.D. (%)	R.S.D. (%)
	5	5	4.3 ± 0.2	85.2 ± 3.9	4.6
Dog plasma	20	5	17.4 ± 1.0	86.8 ± 5.5	6.3
	80	5	71.9 ± 3.6	89.8 ± 5.0	5.6

Table 3
Pharmacokinetic parameters of buagafuran in dogs after single-oral doses (8, 16, and 32 mg/kg, $n = 6$)

Dose (mg/kg)	$AUC_{0-\infty}$ (μg h/l)	C_{max} (μg/l)	T_{max} (h)	MRT (h)
8	11.3 ± 2.2	5.8 ± 1.3	0.3 ± 0.1	1.7 ± 0.5
12	84.9 ± 27.2	28.9 ± 15.2	0.4 ± 0.1	4.3 ± 1.2
32	191.3 ± 80.2	68.6 ± 44.5	0.7 ± 0.4	6.1 ± 2.3

Table 1
Intra- and inter-day precision and accuracy of GC–ToFMS analysis of buagafuran in dog plasma

Concentration (ng/ml)	Intra-day			Inter-day		
	Mean ± S.D. ($n = 5$)	Precision (% CV)	Accuracy (% bias)	Mean ± S.D. ($n = 5$)	Precision (% CV)	Accuracy (% bias)
5	5.2 ± 0.2	3.4	4.8	4.7 ± 0.6	13.3	−6.8
20	20.4 ± 1.0	4.8	1.8	20.1 ± 1.4	7.1	0.4
80	84.4 ± 3.6	4.3	5.5	80.4 ± 11.0	13.7	0.5

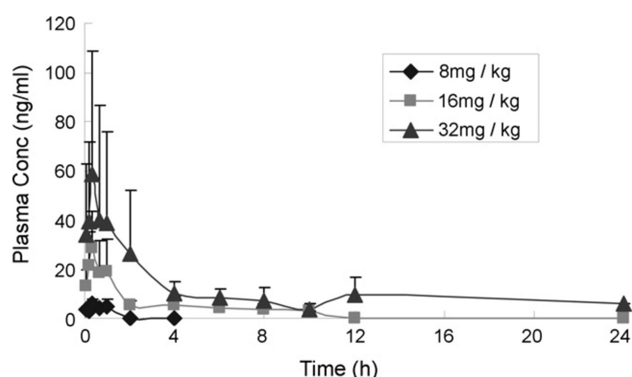

Fig. 3. Mean plasma concentration–time profiles of buagafuran after single-oral doses (8, 16 and 32 mg/kg) in dogs ($n = 6$).

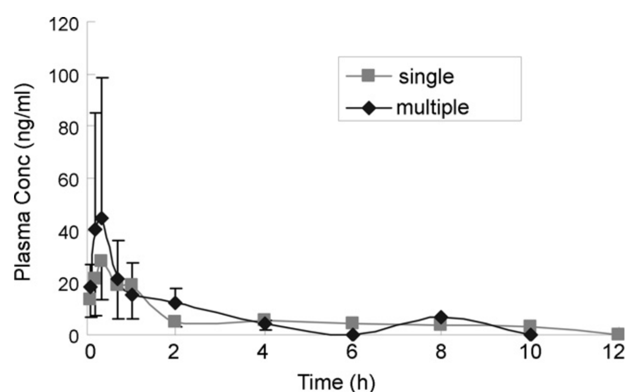

Fig. 4. Comparing the mean plasma concentration–time profiles of buagafuran after single and multiple doses (16 mg/kg) in dogs ($n = 6$).

Table 4
Pharmacokinetic parameters of buagafuran in dogs after single- and multiple-oral doses (16 mg/kg, $n = 6$)

Dose (mg/kg)	$AUC_{0-\infty}$ (μg h/l)	C_{max} (μg/l)	T_{max} (h)	MRT (h)
Single dose	84.9 ± 27.2	28.9 ± 15.2	0.4 ± 0.1	4.3 ± 1.2
Multiple doses	64.4 ± 42.5	48.9 ± 49.3	0.3 ± 0.1	1.9 ± 0.7

half-life of buagafuran. The peak of buagafuran in blood reached rapidly at 0.33, 0.39 and 0.72 h and eliminated from plasma with a MRT of 1.68, 4.33 and 6.14 h after dosing of 8, 16 and 32 mg/kg, respectively. The C_{max} and $AUC_{0-\infty}$ increased proportionally with the increase of doses, indicating the linear pharmacokinetics of buagafuran in dogs from 8 to 32 mg/kg.

The comparision of mean plasma concentration versus time profiles and pharmacokinetic parameters for buagafuran after single and multiple dosing were shown in Fig. 4 and Table 4. After seven daily doses of buagafuran (16 mg/kg), the increasing of C_{max} (69%), decreasing of $AUC_{0-\infty}$ values (24%) and MRT (57%) were observed compared with single dosing, suggesting that there was a potential inductive effect of buagafuran on the metabolism of itself in dogs. In our previous studies, buagafuran was found to be both substrate and inductor of CYP3A and CYP2E1, which may partly explain the self-induction of buagafuran after multiple doses. These aspects will be subject for another paper.

4. Conclusion

The newly developed GC–ToFMS method provides a sensitive, reproducible and validated assay for the determination of buagafuran in plasma. The present method will help in further studies for characterizing the clinical pharmacokinetics profile of buagafuran in human plasma.

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