

Research Article

Determination of Muscone in Rats Plasma following Oral Administration of Artificial Musk: Using of Combined Headspace Gas Chromatography-Mass Spectrometry

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To develop an analytical method for determination of plasma concentrations of muscone in rats following oral administration of artificial musk, with the aim of investigating the pharmacokinetic profile of artificial musk. Plasma samples were pretreated with acetonitrile to precipitate proteins. Headspace injection coupled with gas chromatography-mass spectrometry was used for quantitative analysis of muscone concentrations. A strong linear relationship was obtained for plasma muscone concentrations ranging from 75.6 to 7560 ng·mL⁻¹ ($R^2 = 0.9998$), with the minimum detectable concentration being 25 ng·mL⁻¹. The within-day and interday precision for determination of three different concentrations of muscone were favorable (RSD < 25%). The average absolute recovery ranged from 83.7 to 88.6%, with an average relative recovery of 100.5 to 109.8%. The method described was characterized by stability and reliability, and in the present study showed significant specificity and high sensitivity. This method would be applicable to the analysis of plasma concentrations of muscone in preclinical contexts, where artificial musk is used.

1. Introduction

Musk is a rare traditional medicine that is widely used in China. However, with the increasing scarcity of natural sources of musk, the use of this rare medicinal material has been strictly restricted, and artificial musk is being promoted as a substitute for the naturally occurring counterpart [1, 2]. Muscone (3-methyl-cyclopentadecanone) is the unique bioactive ingredient in artificial musk [3, 4].

It has cardiac effects and weak anti-inflammatory activity, may also cause excitation of the respiratory and central nerve systems, and increases blood flow in the coronary arteries, explaining its common application for the treatment of coronary heart disease in clinical settings [3–8].

Muscone is quickly metabolized in the human body following absorption through the gastrointestinal tract, with

very low plasma concentrations of the prototype drug. Furthermore, artificial musk has an extremely low content of muscone, making it more difficult to determine plasma levels of muscone [3, 9].

Currently, investigations of muscone focus largely on the pharmacological effects following oral administration and intravenous injection, and gas chromatography is usually used to determine muscone concentrations [4, 10]. However, data on the determination of muscone levels in biological samples following metabolism of artificial musk has not been available. In light of the volatility of muscone, this study is the first to combine headspace injection with gas chromatography-mass spectrometry (GC-MS) for determination of the muscone content of biological samples. The aim of this work was to develop an efficient and reliable analytical method for pharmacodynamic studies of artificial musk.

2. Material and Methods

2.1. Animals, Instrumentation, and Drugs. Animals: male Sprague Dawley rats were purchased from the Jiangning Qinglongshan animal cultivation farm (Nanjing, China) and fed at the Nanjing University of Chinese Medicine. The animal experimental protocols are in accordance with "Guide for the Care and Use of Laboratory Animals" (NIH Publication, revised 1996, number 86-23). The mass spectrometer (Agilent, Santa Clara, CA, USA) was connected to a gas chromatograph (Agilent) equipped with a G1888A headspace sampler (Agilent). Data acquisition and processing was performed on 5975 GC-MSD Chemstation (Agilent). Other instruments used included a 5810R automated high speed refrigerated centrifuge (Eppendorf), a XK96-A quick mixer, and Transferpette pipettes (Germany).

Commercially available artificial musk was obtained from Beijing Lianxin Pharmaceutical Company Ltd. (approval number: Z20040042; lot number: 0711169). The control muscone was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (lot number: 0719-200511). Cyclohexanone (purity 99%) was used as the internal standard. Acetonitrile was HPLC grade (Merk, Darmstadt, Germany). Sodium chloride solution (1%) was prepared in the laboratory.

2.2. Preparation of Standard Muscone Solution. Control standard muscone (0.0567 g) was accurately weighted and dissolved in 100 mL of methanol, giving a muscone stock solution of 567 $\mu\text{g}/\text{mL}$. This solution was serially diluted to give standard solutions of 0.756, 1.134, 5.67, 11.34, 56.7, and 113.4 $\mu\text{g}/\text{mL}$, which were stored in a refrigerator at 4 °C before use in experiments.

2.3. Headspace Injection. The samples to be tested were placed in 10 mL headspace vials with closures and then agitated violently three times for extraction. The key parameters for headspace extraction were as follows: heating temperature 95 °C, equilibrium time 3 min, quantitative loop temperature 110 °C, transmission line temperature 120 °C, filling time of quantitative loop 0.2 min, equilibrium time of quantitative loop 0.1 min, headspace helium pressure 7.1 psi, and sample load 100 μL .

2.4. GC-MS Conditions. The chromatographic conditions were column, HP-5MS 5% Phenyl Methyl Siloxane (30.0 m \times 250 μm \times 0.25 μm); MSD detector; high purity helium (purity > 99.999%) as carrier gas; helium flow, 1.0 mL/min; split ratio, 10 : 1; injection temperature, 220 °C; auxiliary channel temperature, 280 °C; and temperature programme, 50 °C for 1 min increasing to 250 °C for 3.5 min (40 °C/min).

The mass spectrometry conditions were electronic ionization as ion source; ion source temperature, 230 °C; and EM voltage, 1.3 kV. The data acquisition model was selective ion detection. The detected ions were 238 m/z for muscone and 98 m/z for cyclohexanone. The residence time was 100 ms with a solvent delay time of 3 min.

3. Results and Discussion

3.1. Determination of Headspace Equilibrium Temperature. The equilibrium temperature of ingredients in the headspace device is directly related to vapor pressure. A high temperature leads to high vapor pressure, and high concatenation of headspace vapor provides high analytic sensitivity. Thus increasing the temperature is one way of improving sensitivity. In order to promote volatilization of muscone from the samples, 1% aqueous NaCl solution was added during plasma extraction. This resulted in the highest equilibrium temperature being less than 100 °C, preventing an increase in vapor pressure due to the boiling and expansion of water, as this probably affects the tightness of the headspace device and measurement accuracy. The headspace equilibrium time was fixed at 3 min, and the sample chromatographic peak areas were examined at equilibrium temperatures of 90, 93, 95, and 97 °C. The results showed that the response values were highest and similar at 95 and 97 °C. However, because 95 °C would be safer, this was selected as the equilibrium temperature for the following experiments.

3.2. Determination of Headspace Equilibrium Time. Because of the low content of muscone in metabolites, each sample was extracted three times in the headspace device to achieve complete extraction, as far as possible, and the response value for muscone remained constant after three extractions. Because multiple extractions were performed, the equilibrium time, which is usually dependent on the diffusion velocity of the molecule from the sample matrix, could not be too long. In these experiments, the chromatographic response values for muscone were examined at an equilibrium temperature of 95 °C and equilibrium times of 2, 3, and 5 min. The results demonstrated that the response value did not increase with an equilibrium time exceeding 3 min, suggesting that the liquid-gas phase equilibrium composition had been reached at 3 min. Therefore, the headspace equilibrium time was standardized at 3 min.

3.3. Treatment of Plasma Samples. Comparative studies were performed of direct injection of plasma samples containing muscone and injection after precipitation of protein from the plasma samples. Although direct injection was convenient, large amounts of sample were required and detection sensitivity was poor because the proteins in plasma were gelatinized upon heating, which prevented the volatilization of muscone from the samples. In light of the high solubility of muscone in acetonitrile and the better results obtained after precipitation of plasma proteins using acetonitrile compared with H₂SO₄, HCl, and 0.3% H₃PO₄ solution or aqueous KH₂PO₄ solution, the use of acetonitrile for plasma protein precipitation was standardized, and the resulting supernatants were injected. Furthermore, water is the ideal solvent for headspace injection due to its reduced interference, and additional tests showed that addition of an appropriate amount of NaCl enhanced detection sensitivity. Therefore, in our experiments, plasma samples were pretreated in accordance with the following protocol. Acetonitrile (300 μL)

TABLE 1: The absolute and relative recovery of muscone from rat serum*.

Spiked concentration (ng/mL)	% absolute recovery \pm SD	% relative recovery \pm SD
378	88.6 \pm 1.9	109.8 \pm 2.9
756	84.5 \pm 1.8	103.5 \pm 2.1
7560	83.7 \pm 3.1	100.5 \pm 2.7

* $n = 5$.

TABLE 2: Intra- and interday precision and accuracy of muscone in rat serum.

Concentration added (ng/mL)	Concentration measured (ng/mL) \pm SD	Accuracy (%)	CV (%)
Intraday reproducibility ($n = 5$)			
75.6	75.38 \pm 7.24	100.51	9.6
378	358.99 \pm 26.57	94.97	7.4
756	732.56 \pm 46.15	96.90	6.3
3780	3595.91 \pm 186.99	95.13	5.2
Interday reproducibility ($n = 5$)			
75.6	74.76 \pm 7.40	99.68	9.9
378	360.91 \pm 29.59	95.48	8.2
756	731.35 \pm 35.84	96.74	4.9
3780	3604.23 \pm 165.79	95.35	4.6

with the internal standard, cyclohexanone (20 μ L), was added to the plasma sample (300 μ L). After mixing and vortexing for 60 s, the samples were centrifuged for 6 min at 6,000 rpm. The resultant supernatant was placed in the 10 mL headspace injection device, 2 mL of 1% NaCl was added, and the device was closed.

3.4. Determination of Chromatographic Conditions. The tested samples were of biological origin and endogenous interference was unavoidable. Investigation of the chromatographic conditions led to the selection of the following temperature program: 50°C for 1 min, increasing to 250°C at 40°C/min, and held at 250°C for 3.5 min. The retention times of muscone and the internal standard, cyclohexanone, under these conditions were approximately 8.1 and 3.90 min, respectively, with good peak shapes. This indicated that the unexpected internal interference had been removed and that the method exhibited satisfactory specificity.

3.5. Evaluation of Detection Specificity. Artificial musk was administrated orally to rats, plasma samples were collected and treated as described previously, and the chromatogram shown in Figure 1(a) was obtained. Muscone standard solution of known concentration was added to a blank plasma sample without internal standard and the chromatogram shown in Figure 1(b) was obtained. The chromatogram shown in Figure 1(c) was obtained from a blank sample of rat plasma (300 μ L) without internal standard. The data showed that the retention times of muscone and the internal standard, cyclohexanone, were 8.1 and 3.90 min, respectively, with good peak shapes, indicating that endogenous interference has been efficiently removed and the method had good specificity (Figure 1).

3.6. Linearity of Standard Curve and Detection Limit. Muscone standard solutions and internal standard (20 μ L) were

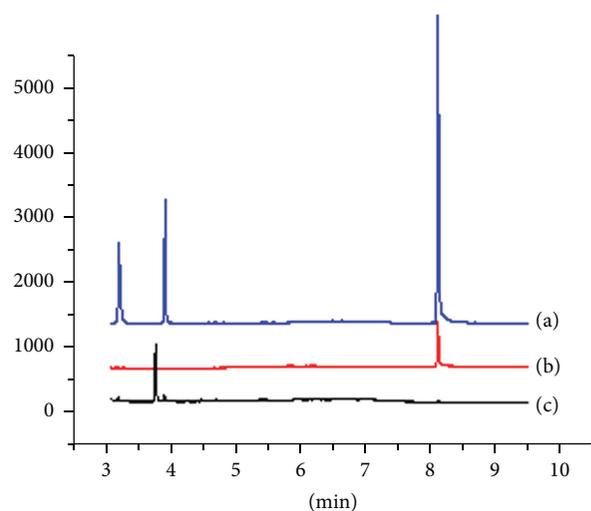


FIGURE 1: Chromatographic evaluation of the specificity of the method for detection of muscone in rat plasma. (a) Rat plasma sample obtained 3 h after oral administration of artificial musk (625 mg·kg⁻¹), with internal standard added; (b) plasma sample from a control rat with muscone (378 ng·mL⁻¹) added; (c) plasma sample from a control rat.

added to control plasma (300 μ L) to give muscone concentrations of 75.6, 378, 756, 3,780, and 7,560 ng·mL⁻¹. The samples were treated as described previously, and headspace injection was performed using the previously established conditions. With the muscone concentration (C , ng·mL⁻¹) as the abscissa and the ratio (f) of the muscone peak area (A_S) to the cyclohexanone peak area (A_i) as the ordinate, the regression equation

$$f = 0.0016C - 0.0059 \quad \left(w = \frac{1}{C}, R^2 = 0.9998 \right) \quad (1)$$

TABLE 3: Method stability of muscone in plasma.

Conditions	Concentration added (ng/mL)		
	378	756	7560
Direction injection	376.2	747.13	7210.94
Room temperature for 6 h	321.97	757.46	7435
Freeze thawing	391.17	696.84	7174
4°C for 10 h	407.91	794.98	8233.1
-26°C for one week	344.53	686.77	8694.88
Accuracy range (%)	85%~108	91~105	95~115

was obtained, based on the weighted least squares method. This data indicated that the standard curve was linear for plasma muscone concentrations ranging from 75.6 to 7560 ng·mL⁻¹, with the minimum detection limit being 25 ng·mL⁻¹ (S/N ratio = 3).

3.7. Precision of Method and Recovery Rate. The recovery rate was calculated using the following equation: recovery rate = (average peak area for tested samples/average peak area for standard solution) × 100%. The relative recovery rate was determined by inserting the peak area ratio for plasma muscone to internal standard into the standard curve equation and then expressing the detected concentration as a percentage of the added concentration. The data (Table 1) demonstrates that the average rate of recovery of muscone ranged from 84.1 to 88.6%, and the RSDs were less than 10%. The results also showed that the relative recovery rates for high, moderate, and low concentrations of muscone were from 100.5 to 109.8%, which met the requirements for analysis of biological samples.

Plasma samples containing four different concentrations of muscone (75.6, 378, 756, and 3780 ng·mL⁻¹) were analysed using a standard curve obtained on the same day, and the sample containing each concentration was analysed six times. This analysis was repeated on three days, and the interday precision and within-day precision (RSD%) were calculated. The results showed that both these parameters were less than 15% (Table 2). In addition, plasma samples containing four different concentrations of muscone were prepared and treated as described previously, and the peak areas for muscone and the internal standard were measured; similarly, the peak areas for muscone and the internal standard were measured after direct injection of plasma samples containing the same three concentrations of muscone. The samples containing each concentration were analysed five times.

3.8. Stability of Muscone in Plasma. Five replicates of plasma samples containing muscone at concentrations of 378, 756, and 7560 ng/mL were prepared. The stability of muscone in these samples was assessed under the following conditions: immediate injection, storage at room temperature for 6 h, three cycles of freeze-thawing, storage in a refrigerator at 4°C for 10 h, and storage in a freezer at -26°C for one week. Analysis by GC-MS with headspace injection demonstrated that muscone was stable in plasma samples stored under all

TABLE 4: Pharmacokinetic parameters of muscone in rat serum after oral administration of artificial musk (625 mg·kg⁻¹, n = 6)*.

Parameter	Estimate (mean ± SD)
t_{\max} (min)	54 ± 2.24
C_{\max} (ng mL ⁻¹)	695.33 ± 68.35
$AUC_{0 \rightarrow \infty}$ (ng min mL ⁻¹)	67456.03 ± 7023.72
$t_{0.5}$ (min)	96.26 ± 0.78
MRT (min)	198.91 ± 23.72

* $AUC_{0 \rightarrow \infty}$, the area under curve concentration-time; C_{\max} , maximum concentration at t_{\max} ; $t_{0.5}$, elimination half life time; MRT, mean residence time.

these conditions, and the accuracy was between 85 and 115% (Table 3).

3.9. Collection and Analysis of Plasma Samples from Rats. Six healthy, male Sprague Dawley rats were deprived of food for 12 h, then artificial musk (625 mg·kg⁻¹) was administered orally, and pharmacokinetic parameters of muscone in rat serum was shown as Table 4. Blood samples were obtained through the eye socket at time points of 5, 10, 20, 30, 45, and 60 min and 1.5, 2, 3, 5, 8, 10, and 12 h after administration. The blood samples were mixed with heparin, and then centrifuged at 5,000 rpm for 15 min and the separated plasma samples were stored at -26°C. The analytical method of GC-MS with headspace injection that was developed was used to determine the muscone concentrations in these plasma samples, and the chromatogram and chromatographic peak areas were recorded. The concentration-time curve for muscone in plasma is shown in Figure 2.

4. Conclusion

Headspace injection combined with GC/MS was used to determine the concentration of muscone in the plasma of rats following oral administration of artificial musk. The plasma samples were pretreated with acetonitrile to precipitate proteins, and the appropriate temperature program, headspace equilibrium time, and temperature were determined. Using these optimized conditions, a reliable detection method was developed, which showed high specificity and sensitivity for the determination of plasma concentrations of muscone, the primary pharmacological constituent of artificial musk. The results showed that the method developed was simple

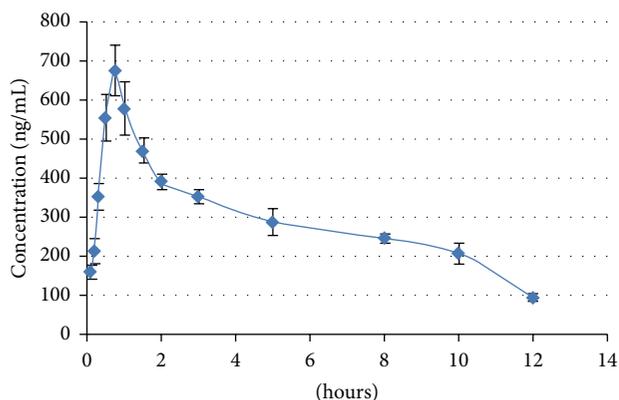


FIGURE 2: Concentration-time curve for muscone in plasma of rats after a single intragastric administration of artificial musk ($625 \text{ mg}\cdot\text{kg}^{-1}$, $n = 6$).

but reliable and applicable to the determination of plasma concentrations of muscone in the preclinical context where artificial musk is used. This method will be useful for pharmacokinetic studies on artificial musk, as well as muscone.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Haitao Li, Yujing Leng, Haishan Deng, and Haibo Cheng contributed equally to this work and are cofirst authors for this paper.

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