Simultaneous determination of borneol and its metabolite in rat plasma by GC–MS and its application to pharmacokinetic study

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Abstract A gas chromatography mass spectrometry (GC–MS) method has been developed and fully validated for the simultaneous determination of natural borneol (NB) and its metabolite, camphor, in rat plasma. Following a single liquid–liquid extraction, the analytes were separated using an HP-5MS capillary column (0.25 mm × 30 m × 0.25 μm) and analyzed by MS in the selected ion monitoring mode. Selected ion monitor (m/z) of borneol, camphor and internal standard was 95, 95 and 128, respectively. Linearity, accuracy, precision and extraction recovery of the analytes were all satisfactory. The method was successfully applied to pharmacokinetic studies of NB after oral administration to Wistar rats.

1. Introduction

Borneol (Fig. 1A), usually used as an important adjuvant in “Su-Xiao-Jiu-Xin-Wan”, “Su-He-Xiang-Wan”, and “Liu-Shen-Wan” for preventing and curing cardiovascular and cerebrovascular diseases [1,2]. It is classified into natural borneol (NB) and synthethetical borneol (SB). Because SB is much easier to get and cheaper than NB, it has been widely used in Chinese formulas. But due to its unstable quality in storage, it can be transformed into camphor and result in higher reproductive toxicity [3–5]. Camphor is reported to cause seizures, respiratory failure, myocarditis and hepatotoxicity; fetuses and infants are specially susceptible to camphor [6–8]. Therefore, NB is becoming more and more widely used. However, there are few reports about its in vivo study, and let alone its metabolite.

Several papers about the pharmacokinetic study of NB have been reported. These results indicate that camphor is the main metabolite of NB, too [9–13]. Therefore, it is necessary to study the pharmacokinetics of camphor after oral administration of NB and to discuss the conversion ration from NB to camphor in vivo for the purpose of safety. In this paper, we hereby developed a systematic gas chromatography mass spectrometry (GC–MS) method for the simultaneous determination of borneol and camphor in rat plasma. It was successfully applied to the pharmacokinetic study of NB in Wistar rats.

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2. **Experimental**

2.1. **Chemicals and reagents**

NB reference standard (purity > 99.9%) and camphor (purity > 99.3%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Naphthalene (purity ≥ 98%, internal standard, IS, Fig. 1C) was purchased from Damao Chemical Reagent Factory (Tianjin, China). NB (99.4% purity, batch no. 20060800) was purchased from Linke NB Factory (Jiangxi, China) for animal experiments.

2.2. **Apparatus and operating conditions**

A Thermo DSQ ITTrace GC–MS system (Thermo Scientific, USA) with Xcalibur station was used in the study. GC separation was accomplished on HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm particle size). The inlet temperature was set at 210 °C. The oven temperature program was started at an initial temperature of 80 °C, lasting for 1.0 min, then increased to 145 °C at 30 °C/min and retained for 3.0 min. Samples were injected in the split mode (1/10), using helium as carrier gas (99.9%, 1 mL/min). In the MS system, both the ion source temperature and inlet temperature were 280 °C. Electron impact ionization (EI) mode was used with nominal electron energy (70 eV). Selected ion monitor (SIM, m/z) of NB, camphor and IS was 95, 95 and 128, respectively. Processing data were acquired from 3.00 to 6.18 min.

2.3. **Preparation of standard and quality control (QC) samples**

The stock solutions of NB (100 μg/mL), camphor (100 μg/mL) and IS (100 μg/mL) were prepared in hexane. A series of working solutions were obtained by diluting stock solutions with hexane. The IS working solution (100 ng/mL) was prepared by diluting stock solution with hexane. All solutions were stored at 4 °C and shielded from light.

Appropriate amounts of NB and camphor working solutions were added to 100 μL blank plasma to yield effective concentration in plasma. Effective concentrations in plasma samples were 15, 30, 150, 300, 1500, 3000 and 4000 ng/mL for NB, while 30, 60, 150, 300, 1500, 3000 and 4000 ng/mL for camphor. The QC samples used in the validation and during the pharmacokinetic study were prepared in the same way as the calibration standard. The three QC concentrations of NB (camphor) were 30 (60), 300 (300) and 3000 (3000) ng/mL.

2.4. **Sample preparation**

20 μL IS (100 ng/mL) working solution and 130 μL hexane were added to an aliquot of 100 μL rat plasma sample; the mixture was then vortex-mixed for 3 min. Then 100 μL supernatant was transferred to glass conical inserts after centrifugation for 10 min at 15,000 rpm (4 °C) and placed into 2 mL brown autosampler vials (22 °C) with screwtop caps and PTFE septa. One microliter of supernatant was injected into the GC–MS system.

2.5. **Method validation**

2.5.1. **Selectivity**

The selectivity of the method was investigated by comparing chromatograms of blank plasma, blank plasma sample spiked with standard at lower limit of quantification (LLOQ) and plasma sample at 0.25 h after oral administration of NB (90 mg/kg).

2.5.2. **Linearity and LLOQ**

The calibration curve consisted of six concentration levels. Each of these samples was prepared and assayed in triplicate on three separate days. The calibration curve was obtained by plotting the peak area ratio (Y) of the analyte to IS versus the nominal concentration (X) of either NB or camphor. These curves were described as Y = a + bx (1/x² weighted). LLOQ was defined as the lowest concentration whose precision (expressed as relative standard deviation, RSD) and accuracy (expressed as relative error, RE) were both ≤ 20%.

2.5.3. **Accuracy and precision**

The accuracy and precision of the method were assessed by analyzing three QC levels samples each in six replicates on three consecutive days. The inter- and intra-day precision is expressed as the relative standard deviation (RSD). Accuracy is defined as the relative error (RE) and is calculated using the formula RE (%) = [(measured value – theoretical value)/theoretical value] x 100%. Both accuracy (RE) and precision (RSD) were expected to be within ±15% as being acceptable.

2.5.4. **Extraction recovery**

Extraction recoveries of NB and camphor were assessed by comparing the peak responses from QC samples to those from post-extracted samples. QC samples were prepared according to previous descriptions; post-extracted samples were prepared as follows: blank plasma samples were extracted and then spiked with standard solutions to yield corresponding equivalent concentrations. Recoveries were tested at three QC levels each in five replicates and should be stable.

2.5.5. **Stability**

Samples at two concentrations levels (150 ng/mL and 3000 ng/mL for NB and camphor) were used in the stability test containing pre-treatment, post-treatment, three cycles of freeze–thaw, and long-term stabilities of the analytes. Pre-treatment stability was assessed by leaving samples at room temperature for 3 h before extraction. For post-treatment assessment, all processed samples were placed into autosampler (22 °C) for 10 h before injection. Freeze–thaw cycle plasma stability test was carried out via repeatedly freezing–thawing samples for three cycles before extraction. Long-term stability was evaluated by storing samples at −20 °C and −80 °C for 24 h before thawing and extraction.

2.5.6. **Pharmacokinetic application of the GC–MS method**

The study was approved by the Animal Ethics Committee of Guangdong Province. Six male Wistar rats weighing 200 ± 20 g...
were purchased from the Center of Experimental Animals, Sun Yat-sen University (Guangzhou, China, Certificate no. SCXK 20110029). The rats were housed in steel cages at 22–26 °C and 50–60% humidity; all animals were allowed free access to tap water but food was withdrawn overnight before the experiment. Rats were administered with a single oral dose (90 mg/kg) of NB suspension in 0.8% carboxymethyl cellulose sodium (corresponding to 0.9 g, the dose a human takes per day). Serial blood samples (0.25 mL) were obtained from the orbital veins at 0, 0.083, 0.167, 0.25, 0.333, 0.5, 0.75, 1, 1.5, 2.5, 4 and 6 h after dosing and immediately centrifuged for 5 min at 15,000 rpm (4 °C) to obtain plasma samples.

The pharmacokinetic parameters were obtained by using Winnonlin 5.0.1 software. \( C_{\text{max}} \) (peak plasma concentration), \( t_{\text{max}} \) (the time to reach peak plasma concentration), and \( \beta \) (elimination rate constant) were determined by linear regression analysis of the logarithmic transformation of the last four data points of the curve. \( t_{1/2\beta} \) (half-time of elimination), \( T_{1/2} \) (0.693/\( k \)), and AUC\(_{0-\infty} \) [the area under the plasma concentration–time curve up to the last time \( t \)] were determined using the trapezoidal rule. AUC\(_{0-\infty} \) (the area under the concentration–time curve) was calculated by adding the value of \( C_t/k \) to AUC\(_{0-t} \). MRT\(_{0-\infty} \) (mean residence time) was equal to the value of AUMC/AUC; AUC\(_{0-\infty} \) ratio was equal to the value of \( ^a\text{AUC}_{0-\infty}/^b\text{AUC}_{0-\infty} \), \( a \): the metabolite camphor and \( b \): the parent drug borneol.

3. Results and discussions

3.1. Optimization of GC–MS conditions

We referred to the gradient temperature program in the study of Huang et al. [14], but changed the last retaining time from 2 min to 3 min. The application of the modified gradient temperature program ensured that the main peaks could be detected in only 6.18 min with excellent resolution. Fig. 2 shows the mass spectra of NB, camphor and IS. According to the relative abundance of each fragment ion in mass spectra, the fragment ion \( m/z \) 95 was used for the analysis of NB and camphor, and \( m/z \) 128 for the analysis of IS. The SIM mode in the study was selected to avoid the analytical interference of endogenous substance and achieve required selectivity.

Fig. 2 The product ion mass spectra, chemical structures monitored transitions of borneol (A) and camphor (B), and naphthalene (C).
Fig. 3  Representative SIM chromatograms for NB and camphor in rat plasma: (A) a blank plasma; (B) a blank plasma spiked with NB (30 ng/mL) and camphor (60 ng/mL) at LLOQ level; and (C) a plasma sample at 0.25 h after oral administration of NB (90 mg/kg).

Table 1  Accuracy, precision (3 days, 6 replicates per day) and recovery (n=5) for NB and camphor in rat plasma.

| Compound | Nominal concentration (ng/mL) | Precision | Accuracy (RE, %) | Recovery |
|----------|-------------------------------|-----------|------------------|----------|
|          |                               | Intra-day | Inter-day        |          |
|          |                               | RSD (%)   | RSD (%)          |          |
| NB       | 30                            | 6.7       | 13.3             | -6.7     | 101.5±6.9 | 6.8     |
|          | 300                           | 4.5       | 14.0             | -8.5     | 99.7±9.3  | 9.3     |
|          | 3000                          | 8.6       | 11.5             | -3.2     | 101.6±6.0 | 5.9     |
| Camphor  | 60                            | 6.7       | 12.8             | -0.7     | 91.6±8.3  | 10.0    |
|          | 300                           | 8.2       | 14.1             | -7.7     | 102.4±9.4 | 10.0    |
|          | 3000                          | 5.8       | 13.7             | -7.8     | 102.3±8.1 | 7.9     |
| IS       | 100                           |           |                  | 83.7±4.7 | 5.6      |

RSD: relative standard deviation; RE: relative error.
3.2. Selectivity, linearity and LLOQ

Fig. 3 shows the typical chromatograms of blank plasma (A), plasma sample spiked with NB, camphor and IS (B), and plasma sample from a rat after oral administration of NB (90 mg/kg) (C). The retention time of camphor, NB and IS was 4.79, 5.04 and 5.33 min, respectively. Blank rat plasma yielded relative clean chromatograms without interfering peaks both to the analytes and IS.

Good linearity was obtained within the range of 15–4000 ng/mL for NB (r = 0.9911) and 30–4000 ng/mL for camphor (r = 0.9937). The LLOQ for NB and camphor was 15 ng/mL with RSD 15.2% and 30 ng/mL with RSD 14.3%, respectively.

3.3. Accuracy and precision

The results of accuracy and precision are shown in Table 1. The intra-day and inter-day precisions (RSD) were less than 14.1% for each QC level of NB and camphor. The accuracy (RE) was within ±8.5% of the target values. These results indicated that it was a precise and accurate method.

3.4. Recovery

As shown in Table 1, the overall recovery efficiency for NB was 94.0% at 30 ng/mL, 93.9% at 300 ng/mL and 91.4% at 3000 ng/mL; the recovery for camphor was 89.5% at 60 ng/mL, 91.0% at 300 ng/mL and 93.0% at 3000 ng/mL; the recovery for IS was 83.7%. The recovery results indicated that hexane was a feasible and appropriate medium for NB, camphor and IS extraction.

3.5. Stability

The stability results (Table 2) indicated that samples were stable on the bench at room temperature for 3 h and at 22 °C environment for 10 h after processing, but not stable in freeze–thaw test and post-freezing stability test in 20 °C and 80 °C environment. It suggested that plasma samples should be processed and determined immediately after obtaining, not be long-term stored or undergo repeated freeze–thaw cycle; processed samples could be placed into autosampler (22 °C) for less than 10 h for batch analysis.

3.6. Application of the developed GC–MS method

According to the provision for NB in the Chinese Pharmacopoeia (Ch.P) [9], the recommended dosage for adults is 0.3–0.9 g. This range of dosage converted for rats is 30–90 mg/kg. We employed 90 mg/kg as dosage administration to rats in consideration of the simultaneous determination for NB and its metabolite camphor.

Previously, gas chromatography (GC) with the flame ionization detection (FID) method was applied to detect NB and menthol in the biomatrix study of Xu et al. [10]. Huang et al. [14] also developed a GC–MS method to investigate NB distribution in mice which referred to the study on the meridian tropism of NB. Li et al. [15] determined the concentration of NB in the brain with a GC–MS method. In this study, we referred to the above papers and developed a GC–MS method to measure the concentration of NB and its metabolite in rat plasma and it has been successfully applied to the pharmacokinetic study of NB and its metabolite.

The plasma profiles of NB and its metabolite, camphor, after oral administration (90 mg/kg) to rats are shown in Fig. 4. For NB, measured concentration ranged from a maximum concentration of 311.35 ng/mL at 0.27 h to a lower concentration of 40.00 ng/mL at 1.5 h and remained stable from 1.5 h to 6 h, which was similar to other previous reports (absorbed rapidly and eliminated rapidly). For camphor, measured concentration reached a maximum level of 150.61 ng/mL at 0.28 h, and the concentration of camphor was below the limit of quantitation after 1.5 h. It indicated that NB was absorbed and metabolized to camphor; meanwhile, camphor could be absorbed rapidly. The concentration of two compounds reached a maximum level at about the same time. The disappearance of the parent drug NB from the plasma was slower with longer MRT of than that of the metabolite, camphor. The AUC∞ ratio of camphor to NB was 0.32, which indicated that NB was metabolized to camphor to a large extent.

RE: relative error.

### Table 2 Stability for natural borneol (NB) and camphor in rat plasma (n = 3).

| Conditions                        | Nominal concentration (ng/mL) | Calculated concentration (ng/mL) | RE (%)   |
|----------------------------------|-----------------------------|---------------------------------|---------|
|                                  | NB     | Camphor | NB     | Camphor | NB     | Camphor |
| Three freeze–thaw cycles         | 150    | 150     | 85.4 ± 2.0 | 75.1 ± 5.0 | −43.1 | −49.9 |
|                                  | 3000   | 3000    | 1470 ± 65  | 1851 ± 130 | −51.0 | −38.3 |
| Post-freezing (−20 °C) for 24 h  | 150    | 150     | 100.0 ± 3.4 | 119.1 ± 8.8 | −33.4 | −20.6 |
|                                  | 3000   | 3000    | 2202 ± 292 | 2378 ± 336 | −26.6 | −20.7 |
| Post-freezing (−80 °C) for 24 h  | 150    | 150     | 101.3 ± 8.4 | 94.4 ± 7.9 | −32.4 | −37.1 |
|                                  | 3000   | 3000    | 2144 ± 179 | 2082 ± 235 | −28.5 | −30.6 |
| Benchtop for 3 h (room temperature) | 150   | 150     | 159.2 ± 13.4 | 154 ± 6.3 | 6.1  | 3.0  |
|                                  | 3000   | 3000    | 2794 ± 33  | 2722 ± 54  | −6.9  | −9.3  |
| Post-preparative (−20 °C) for 24 h | 150  | 150     | 144.5 ± 4.3 | 142.2 ± 6.7 | −3.7  | −5.2  |
|                                  | 3000   | 3000    | 2880 ± 46  | 2883 ± 56  | −4.0  | −3.9  |
camphor accumulation and cause neuronal damage in the cortex and basal ganglia. Pharmacokinetic parameters are summarized in Table 3.

### Table 3 Pharmacokinetic parameters of NB and camphor after oral administration of NB (90 mg/kg) in Wistar rats (mean ± SD, n = 6).

| Parameter                  | NB            | Camphor       |
|----------------------------|---------------|---------------|
| $C_{\text{max}}$ (ng/mL)  | 311.35 ± 192.09 | 150.61 ± 17.50 |
| $t_{\text{max}}$ (h)      | 0.27 ± 0.08   | 0.28 ± 0.05   |
| β ($h^{-1}$)              | 0.76 ± 0.61   | 0.72 ± 0.12   |
| $t_{1/2\beta}$ (h)        | 1.33 ± 0.78   | 1.01 ± 0.09   |
| $AUC_{0\rightarrow t}$ (h ng/mL) | 519.17 ± 75.95 | 125.16 ± 36.11 |
| $AUC_{0\rightarrow \infty}$ (h ng/mL) | 568.97 ± 82.35 | 192.16 ± 37.91 |
| $MRT_{0\rightarrow \infty}$ (h) | 5.50 ± 0.85   | 1.55 ± 0.38   |
| $AUC_{0\rightarrow \infty}$ ratio | 0.32 ± 0.07   |               |

SD: standard deviation.

4. Conclusions

A sensitive and selective GC–MS method for the simultaneous determination of NB and camphor in rat plasma was developed and validated. The proposed method showed appropriate accuracy and precision and was successfully applied for analysis of NB and camphor in rat plasma after oral administration of NB, which indicated that the method is suitable for pharmacokinetic study of NB and camphor.

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