Journal of Chromatography B, 905 (2012) 133–136

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

A gas chromatography–mass spectrometry assay to quantify camphor extracted from goat serum

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ARTICLE INFO

Article history:
Received 9 May 2012
Accepted 31 July 2012
Available online 7 August 2012

Keywords:
Camphor
Gas chromatography–mass spectrometry
Goat serum
Juniper
Monoterpene

ABSTRACT

A sensitive gas chromatography–mass spectrometry (GC–MS) method was developed and validated for quantification and pharmacokinetics of camphor, a major monoterpene of juniper plant, in goat serum. Camphor and internal standard (terpinolene) eluates from solid phase extraction (SPE) with ethyl acetate yielded well resolved peaks and were clearly identified in total and selected ion chromatograms. The elution and injection volumes were optimized for improved detection and quantification of camphor based on peak shape, signal to noise ratio, recoveries, and repeatability. The matrix calibration curve with the good linearity (R^2 = 0.998) and response in the range of 0.005–10.0 μg/mL was used to determine camphor concentration in goat serum. The GC–MS method offered sufficiently low limits of detection (1 ng/mL) and quantitation (3 ng/mL) for camphor concentration in goat serum for the pharmacokinetic study. The proposed method showed good intra- and inter-day variation with relative standard deviation (RSD) of 0.2–7.7% and produced good recovery (96.0–111.6%) and reproducibility (1.6–6.1%) at all spiked levels. Using this method on serum samples obtained from two goats orally dosed with camphor confirmed that the method is suitable for camphor studies in animals.

1. Introduction

Juniper infestation and encroachment poses negative impacts on livestock production, biodiversity of species, water availability and quality, and wildlife habits, and has become most important ecological and economical issues of range management and research [1–3]. The identification and taxonomic classification of the juniper species and subspecies are challenging due to great variation and complexity of the genus *Juniperu*. Juniper produces volatile oils, a class of secondary compounds, as defense mechanisms against grazing, affecting its consumption by animals such as goats and sheep. Goats limit their intake to minimize the potential toxic effects due to the presence of these volatile oils, which include the monoterpenes [4]. Monoterpenes are detrimental to rumen microbial populations and causes digestive sickness, consequently influencing feeding patterns and intakes of goats [2,5]. Camphor is one of the major monoterpenes produced by juniper and can be used as a target molecule to monitor monoterpene consumption in animals.

Although a variety of techniques have been developed for determination of precise amount of monoterpenes in blood and rumen fluid of animals, the sensitivity and the recovery of such techniques are rather erratic and unreliable at low concentrations in blood for biological and pharmacokinetic studies [6–8]. Accurate and precise methods to determine the camphor concentration in the animal blood are of critical value to understand the pharmacokinetics of camphor.

Gas chromatography (GC) is the major chromatographic technique for analysis of monoterpenes from diverse plants and industrial matrices because of the volatile nature of terpenes [7,9,10]. Coupled with different detectors, GC technique presented a powerful way to analyze camphor both qualitatively and quantitatively. Gas chromatograph-flame ionization detection method (GC-FID) combined with solid phase extraction (SPE) has been developed for quantification of monoterpenes in honey and animal plasma at low residue levels [7,10]. The method by Kimball et al. [7] reported a limit of detection (LOD) of 0.25 μg/mL for camphor. However, lower concentration levels than the detection limits established for previous methods might be encountered in some pharmacokinetic studies [11] and comparison studies will require

Abbreviations: BW, body weight; GC-FID, gas chromatograph-flame ionization detection; GC–MS, gas chromatography–mass spectrometry; IS, internal standard; LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation; S.D., standard deviation; SIM, selected ion monitoring; S/N, signal to noise; SPE, solid phase extraction.

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http://dx.doi.org/10.1016/j.jchromb.2012.07.039

a detection method with lower limit of detection (LOD) and limit of quantitation (LOQ). Coupling of gas chromatography and mass spectrometry (GC–MS) has provided a supreme way to achieve high sensitivity and low limit of detection in identifying camphor in some matrices [12,13].

The main objective of this study was to develop and validate a GC–MS method for quantification of camphor in goat serum with optimizing analytical conditions through determination of LOD, LOQ, linearity, specificity, and sensitivity that could be utilized for pharmacokinetic or toxicity studies.

2. Experimental

2.1. Materials

Camphor and terpinolene (internal standard) were obtained from Sigma–Aldrich (St. Louis, MO). High-performance liquid chromatographic (HPLC)-grade ethyl acetate and methanol used for preparation of standard stock solutions were purchased from Fisher Scientific (Fair Lawn, NJ). Solid phase extraction (SPE) cartridges, 500 mg C18, were obtained from Biotage (Biotage, Charlotte, NC).

2.2. Preparation of stock solutions

Stock standard solutions of camphor (100 μg/mL) and terpinolene (862 μg/mL) were prepared by dissolving appropriate quantity of the compounds in ethyl acetate or methanol. These stock solutions were further diluted with the same solvents to 1 μg/mL of camphor and 8.62 μg/mL of terpinolene for use in calibration standards, precision and accuracy replicates, and spiking samples.

2.3. Extraction procedure

A C18 SPE cartridge equipped with a 10-mL solvent reservoir was pre-conditioned with 5 mL of methanol and followed with 10 mL of deionized water wash. The conditioned SPE was loaded with 4 mL of serum sample and placed under a vacuum pressure of approximately 20 Pa and followed with 10 mL of water wash. The SPE cartridge was dried under vacuum for 10 min and eluted with ethyl acetate. In order to test the eluting efficiency, the absorbed analytes in the SPE column was eluted with different volumes (1, 2, 3, and 4 mL) of ethyl acetate at 0.005, 0.03, 0.5, 1.0, and 5.0 μg/mL levels of camphor to determine optimal elution volumes for maximum recoveries and fine chromatograms.

2.4. Gas chromatography–mass spectrometry (GC–MS) analysis

The elute from SPE column was injected into the gas chromatography–mass spectrometry (GC–MS) using a Combi PAL autosampler (Agilent Technologies, Santa Clara, CA). For optimization of injector performance and improvement of method sensitivity, the different injection volumes of 1, 2, 3, and 4 μL were tested at 0.1 and 1.0 μg/mL of camphor under a splitless injection mode.

GC–MS analysis was run on an Agilent 7890 GC (Agilent Technologies) coupled with an Agilent 5975 inert mass spectrometer. The GC analytical column was a HP-5MSI column at 30-m × 0.25-mm I.D., 0.25 μm film thickness. The temperature of injection port was set at 280 °C, whereas that of MS transfer line temperature was 100 °C. An optimal volume of sample was injected with an injection rate of 50 μL/s by the autosampler. The GC oven temperature was programmed as follows: initial temperature 40 °C for 0.5 min, 40–110 °C at 5 °C/min, and 110–300 °C at 20 °C/min, requiring a total run time of 24 min. Helium was a carrier gas delivered at a constant flow rate of 1.0 mL/min and inlet pressure of 7.0 psi. MS conditions were set to simultaneously acquire scan and a selected ion monitoring (SIM) data for quantification of camphor. Ions were monitored at retention time of 12.7 min (*m*/*z* 91, 93, 121, and 136) for terpinolene, and at 14.2 min (*m*/*z* 81, 95, 108, 152) for camphor. The raw chromatography and mass spectrum data were processed with Enhanced Chemstation (Agilent Technologies).

Table 1
Responses of analytes corresponding to changes in injection volume.[a]

Camphor concentration (μg/mL)	Injection volume (μL)	S/N ratio[b]	RSD (%)[c]
0.1	1	6.7 ± 1.0	11.0
	2	13.9 ± 0.9	4.5
	3	17.0 ± 1.7	1.2
	4	41.7 ± 3.9	3.3
1.0	1	41.4 ± 3.6	1.8
	2	113.4 ± 10.0	4.8
	3	133.1 ± 9.1	2.6
	4	416.3 ± 20.2	1.4

[a] Total number of measurements for each injection volume (n) = 5.
[b] Signal-to-noise ratio.
[c] Relative standard deviation (RSD, %) of the ratio of camphor to internal standard (terpinolene) areas.

2.5. Analytical characteristics and validation of GC–MS method

Blank goat serum spiked with camphor and internal standard was loaded to the SPE columns to prepare matrix calibration standards as described above. The concentrations of matrix calibration standards are in the range from 0.005 to 10 μg/mL, while those for the precision and accuracy replicates were 0.01, 0.05, 0.1, 0.5, 1.0, and 5.0 μg/mL. The ratio of the peak area of camphor to internal standard vs camphor concentration was plotted to obtain the standard curve using equal-weighted linear regression analysis. The camphor in serum samples was determined using the slope of the standard curve. The sensitivity of the method was assessed by determining the limit of detection (LOD) and the limit of quantification (LOQ) defined as the concentrations with a signal-to-noise (S/N) of 3 and 10, respectively.

2.6. Pharmacokinetics of camphor in goat

Goat serum samples, for method validation, were obtained from goats that had not grazed in pastures containing juniper for at least 14 days. Two Boar-Spanish goats, approximately 1 year of age, were used in the animal dosing portion of the study. Goats were fasted the night before the dosing treatment. Goats were dosed with camphor, 200 mg/kg BW, via a gastric lavage tube in a single bolus. Blood samples (10 mL each) were collected from the jugular vein into glass collecting tubes containing no additives (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ). The 10 mL of blood were taken prior to dosing (the blank serum sample) and at 10, 20, 30, 45 min and 1, 1.25, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 h after dosing. Samples were stored at room temperature for 2 h and then centrifuged at 2000 x *g* for 15 min. The serum was collected and stored for less than 24 h at 4 °C until used in the extraction procedure. The study was approved by the Institutional Animal Care and Use Committee of Texas A&M University.

3. Results and discussion

3.1. Optimization of the GC–MS conditions

The larger injection volumes at injection well produced better peak shape, higher signal-to-noise (S/N) ratio, and better overall repeatability (Table 1). With an increase of the injection volume, the S/N ratio of camphor improved at both concentrations of

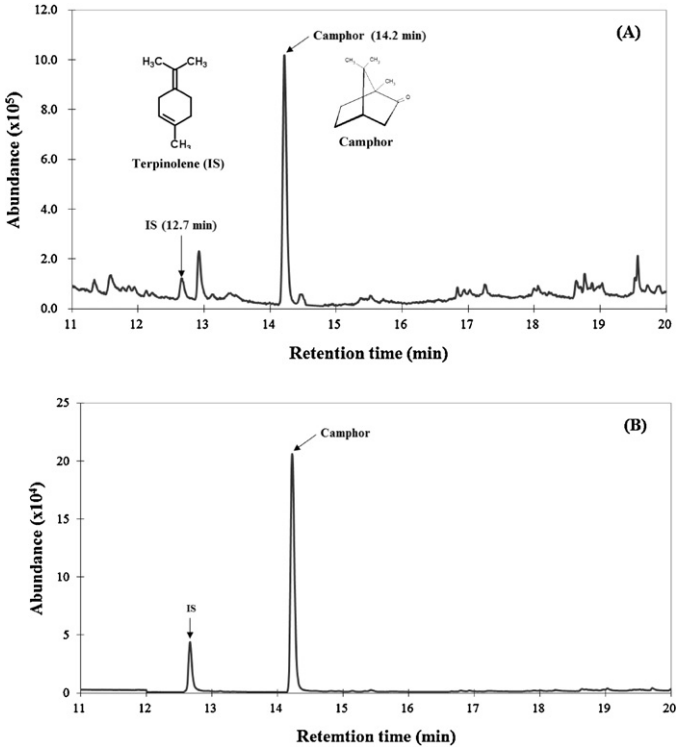

Fig. 1. GC–MS total ion chromatogram (A) and selected ion chromatogram (B) of camphor (0.5 μg/mL) and internal standard (IS, 0.1 μg/mL) in a goat serum.

0.1 and 1.0 μg/mL. In addition, RSDs of the response area ratio were generally lower at larger injection volumes, indicating better repeatability of the response at larger injection volume. As a result, a 4 μL of injection volume was employed for subsequent experiments in the present study.

The optimal volume of extracting solvent was determined at which camphor and internal standard in goat serum samples are fully extracted. The recovery percentages were not significantly different among different elution volumes at each concentration while the coefficient of determination (R^2) was slightly lower at 1 mL (Table 2). The subsequent second extraction revealed that camphor and internal standard were less completely removed from SPE column by the first extraction with 1 mL ethyl acetate compared to higher volumes. The 3 and 4 mL improved removal of two analytes with acceptable accuracy or precision. However, these volumes are likely to increase the analysis time and thus limit the number of samples in a certain time period. Therefore, the final volume of the SPE extract was determined as 2.0 mL for this study.

3.2. Selectivity

Fig. 1 shows the total ion chromatograms (A) and selected ion chromatogram (B) of goat serum samples spiked with camphor and internal standard. The proposed method yielded well resolved peaks, allowing clear identification of two analytes. No interference peak was observed in any matrix standard and goat serum samples at the retention times of camphor and internal standard. The most abundant ions of camphor and internal standard were the fragmentation ion at m/z 95 and 93, respectively and the ratio of two ions was used for the quantitative determination of camphor. The m/z 95 fragment corresponding to a dimethylcyclopentenyl ion is formed from loss of carbon atoms followed by a selective loss of methyl groups from camphor molecule [14,15].

3.3. Linearity

The good linearity of the method was observed in the range of tested concentrations. The calibration curve for camphor was $Y = 16.78x + 0.023$ ($R^2 = 0.998$), where Y is the ratio of camphor and internal standard peak area and x is the camphor concentration, μg/mL. Comparison showed that the slope and correlation coefficients of the calibration curves obtained from the internal standard method were better than those from the external calibration method (data not shown).

3.4. Sensitivity

The limit of detection (LOD) for camphor in serum was 1.0 ng/mL and the limit of quantitation (LOQ) was 3.0 ng/mL. This was a significant improvement compared with the previous report by Kimball et al. [7]. In that study, the camphor concentration was much higher (0.250–5.00 μg/mL) than the ng/mL level. For the benefit of the pharmacokinetic study, the GC–MS method developed in this work offers a more accurate way to estimate and monitor the camphor concentration in the animal.

3.5. Recovery and reproducibility

The recovery and RSD of camphor in goat serum were consistent at all fortification levels (0.01, 0.03, 0.1, 1.0, and 10.0 μg/mL) tested in this study. The obtained recoveries of camphor were in the range of 96.0–111.6% while the range of RSD for the method was between 1.6 and 6.1%. However, a specific recovery pattern was not observed. Serum recoveries of camphor obtained from this study are comparable to those found in previous studies [7,8,10].

3.6. Precision and accuracy

The intra- and inter-day RSDs for camphor vary with levels of camphor (Table 3). All intra-day RSDs were within 8.0%, ranging from 0.2 to 7.4%. Although the variation was not decreased proportionally to camphor concentration, smaller variation was generally observed at higher concentrations. The inter-day RSDs were less than 10% at all levels of camphor. The values for method accuracy were positive or negative with the range from −0.7 to 10.6%. These observations indicate that the proposed method has acceptable precision and accuracy for camphor at μg/mL and ng/mL levels.

Table 2
Effect of volume of extracting solvent on recovery percentages of camphor spiked into SPE cartridge.

Elution volume (mL)	Recovery (%)[a]					Coefficient of determination (R^2)
	0.005 μg/mL	0.03 μg/mL	0.5 μg/mL	1.0 μg/mL	5.0 μg/mL	
1	103.2	124.6	137.3	114.4	99.0	0.9965
2	110.8	127.5	106.1	96.6	100.0	0.9999
3	98.6	121.5	103.8	94.4	100.1	0.9998
4	103.4	114.5	115.6	93.4	100.0	0.9994

[a] Recovery (%) at each elution volume was calculated using the calibration curve made with the spiked samples used for extraction in the range of 0.005–5.0 μg/mL.

Table 3
Precision and accuracy of the method for camphor in goat serum.

Concentration spiked to goat serum (μg/mL)	Intra-day				Inter-day	
	Estimated concentration (μg/mL) (%RSD)[a]				Estimated concentration (μg/mL) (%RSD)[b]	Accuracy (%)[c]
	Day 1	Day 2	Day 3	Day 4		
0.01	0.010 (6.6)	0.011 (2.4)	0.010 (7.4)	0.010 (1.8)	0.010 (4.9)	2.3
0.05	0.057 (0.4)	0.058 (4.0)	0.058 (6.0)	0.049 (4.7)	0.055 (7.7)	10.6
0.1	0.104 (3.2)	0.102 (7.2)	0.114 (1.7)	0.109 (2.9)	0.109 (5.6)	8.7
0.5	0.493 (0.5)	0.505 (2.3)	0.501 (3.1)	0.490 (3.1)	0.507 (3.8)	1.4
1.0	1.014 (0.2)	1.057 (3.7)	1.045 (2.2)	1.002 (1.8)	1.031 (3.0)	3.1
5.0	4.850 (2.6)	5.068 (2.6)	4.697 (1.3)	5.235 (2.1)	4.967 (4.7)	−0.7

[a] Mean and relative standard deviation (%RSD) of three measurements ($n = 3$).
[b] Mean and relative standard deviation (%RSD) of twelve measurements ($n = 12$).
[c] Accuracy (%) = [(estimated − spiked)/spiked] × 100.

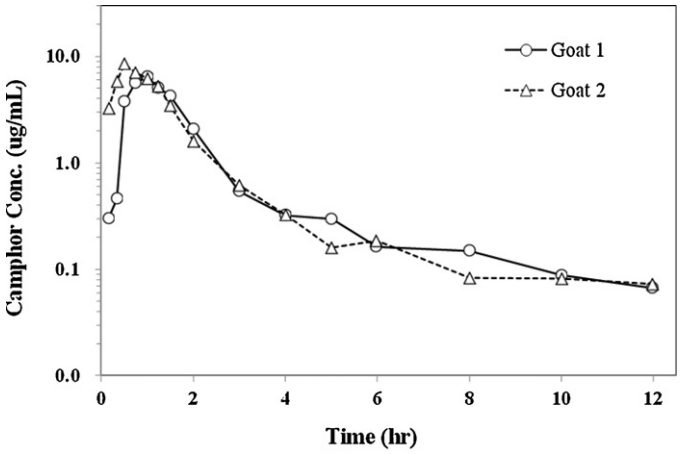

Fig. 2. Serum concentration–time profile following oral administration of camphor at 200 mg/kg body weight (BW) to boar-Spanish goat crosses.

3.7. Application in pharmacokinetics

Two serum concentration–time profiles of camphor in goat after the oral administration of camphor 200 mg/kg body weight (BW) are illustrated in Fig. 2. Goat 1 showed a lower camphor level (0.3 μg/mL) than goat 2 (3.2 μg/mL) at 10 min after dosing. In goat 1, measured concentrations ranged from a maximum concentration of 6.4 μg/mL at 1 h to a minimum concentration of 0.07 μg/mL at 12 h. In goat 2, measured concentrations ranged from a maximum concentration of 8.5 μg/mL at 30 min to a minimum concentration of 0.07 μg/mL at 12 h. One reason for the difference in the maximum concentration and its corresponding time between two subjects might be attributed to the presence of food in rumen and rumen microbes adapting to camphor, affecting the degradation and absorption of this monoterpene [2,16,17]. After the peak serum concentration, camphor declined bi-exponentially in both goats. At 6 h post dosing, only residual concentrations of camphor in serum were found in both subjects. The levels of camphor in goat, particularly after oral administration of low concentrations, may be less than 10 ng/mL. During the entire 12-h interval in this study, no serum concentration of camphor was below the limits of detection and quantitation.

4. Conclusions

In the present study, a simple, rapid, and reliable GC–MS method was developed for detection and quantitative analysis of monoterpene camphor in goat serum, using terpinolene as the internal standard. The results obtained from the validation of this method indicate that the method is adequately precise and accurate for determining camphor in goat serum at low concentration levels. The proposed method clearly showed a feasibility and practicability to examine the effect of dose on the adsorption and elimination of camphor from bloodstream, which should provide the biological and pharmacokinetic data to develop animal models for identification of camphor tolerant and sensitive goats. We also anticipate little difficulty to assay other monoterpenes in animals grazing juniper plants, using this analytical method. The proposed method would be fundamental to the research and project to develop the strategies for managing rangelands dominated by juniper and other monoterpene containing plants and trees.

Acknowledgments

The authors acknowledge that this research was supported in part by The Texas Department of Agriculture Food and Fibers Commission and Texas AgriLife Research and Extension.

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