Chromatographic Analysis of Sagebrush Monoterpenes in Blood Plasma

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Abstract

An analytical method is required for the analysis of monoterpenes in animal plasma to support a pharmacokinetic study. Monoterpenes common to sagebrush are extracted from sheep plasma by employing solid-phase extraction (SPE), followed by analysis of the extracts by gas chromatography with flame ionization detection. The analytes are quantitated versus an external standard and by comparison with a surrogate standard added to the sample prior to extraction. In addition to comparing the two quantitative methods, the storage stability of the analytes in plasma and SPE columns is evaluated. Both methods employed for quantitation yield precision suitable for pharmacokinetic studies. However, determination of monoterpenes residues versus external standards produces improved accuracy as compared with use of the surrogate standard. Some analyte loss is observed from plasma samples stored for five weeks at –12°C. However, storage of extracts on the SPE columns affords excellent stability.

Introduction

Sagebrush (*Artemesia* spp) provides essential winter forage for domestic and wild herbivores in the rangelands of the western United States (1,2). Though the abundance of sagebrush-steppe decreased substantially during the past century, the density of sagebrush has generally increased because of selective grazing and fire suppression, both of which reduce plant diversity (3). Managed grazing is an economical and environmentally acceptable method to control sagebrush. However, sagebrush contains several monoterpenes that serve as antifeedants for many mammalian herbivores (4,5). Sheep feeding on sagebrush ingest large amounts of terpenes. To reduce the possibility of ingesting lethal quantities of terpenes, sheep must regulate intake of terpenes below certain critical thresholds (6,7). However, regulation of intake sets a limit on the amount of daily food consumption.

The physiological mechanisms that influence how herbivores limit consumption of monoterpene-containing plants are not well understood. Pharmacokinetic studies are required to elucidate those mechanisms, and analytical methods capable of precise quantitative determinations of monoterpenes in physiological systems must be available for such studies. As a first step toward determining how herbivores regulate intake of sagebrush monoterpenes, a study was designed to intravenously deliver terpenes to lambs while measuring certain behavioral, physiological, and chemical responses. Chief among these was the measurement of monoterpene residues in the bloodstream. The monoterpenes of interest were camphor, 1,8-cineole, and *p*-cymene. These monoterpenes are abundant in sagebrush and have been implicated in deterrence of herbivory (4,8).

Gas chromatographic (GC) techniques for the analysis of monoterpenes from a variety of plant and industrial matrices are well known (9). A recent method described the use of solid-phase extraction (SPE) for the direct isolation of 1,8-cineole and camphor (among others) from a complex liquid sample (honey) followed by GC analysis with flame ionization detection (FID) (10). Correspondingly, a method for the analyses of camphor, 1,8-cineole, and *p*-cymene in sheep plasma by SPE–GC–FID was developed. Analyte recovery following storage of fortified plasma samples and SPE columns for 35 days prior to GC analyses was evaluated. Further, the results yielded by quantitative analyses using external standard calibration and direct quantitation versus a surrogate standard were compared.

Experimental

Camphor (CAS# 464-19-3), 1,8-cineole (470-82-6), *p*-cymene (99-87-6) and Intralipid (20% emulsion) were purchased from Sigma-Aldrich (Milwaukee, WI). Terpinolene (CAS# 586-62-9) was obtained from TCI America (Portland, OR). Standard monoterpene solutions were prepared in high-performance liquid chromatographic (HPLC)-grade ethyl acetate (Fisher Scientific, Fair Lawn, NJ) and fortification solutions were prepared in HPLC-grade methanol (EM Science, Gibbstown, NJ). Control sheep plasma was obtained by drawing blood from adult sheep using an indwelling catheter placed in the jugular vein. Blood was collected in heparinized tubes and plasma separated by centrifugation. This study was conducted according

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to procedures approved by the Utah State University Institutional Animal Care and Use Committee.

Isolute SPE columns (C18, 500-mg sorbent, 10-mL reservoir) were employed for the extractions (International Sorbent Technology, Wales, UK). The chromatographic system consisted of a Hewlett-Packard 5890 GC (Agilent Technologies, Avondale, PA) equipped with an FID. The analytical column was a 30-m × 0.25-mm DB-5.625, 0.25 µm (J&W Scientific, Folsom, CA).

SPE columns were conditioned with 5 mL of methanol, followed by 10 mL of water. Three milliliters of plasma were passed through the column, followed by 10 mL of water (column wash). The columns were allowed to dry under vacuum for 10 min and then immediately eluted with 1.00 mL of ethyl acetate or stored for later elution. The column eluate was placed in an autosampler vial for injection into the GC. A final extract volume of 1.00 mL was assumed for quantitative analyses, regardless of the volume actually recovered from the SPE column.

One-microliter splitless injections (split time of 1.0 min) were made under the following conditions: the injection temperature was 200°C and the detector temperature was 325°C. The initial oven temperature of 40°C was held for 0.5 min. The first oven ramp took the oven to 110°C at a rate of 5°C/min (0-min hold time). The final ramp of 20°C/min took the oven to its final temperature of 300°C (0-min hold time). The total run time was 24 min. The carrier gas was helium delivered at a constant 39 cm/s by employing electronic pressure control. The detector gases were hydrogen (30.0 mL/min), nitrogen (make-up, 30.0 mL/min), and air (400 mL/min).

Detector responses were evaluated for each analyte and the surrogate standard (terpinolene) over the range of 0.250–5.00 µg/mL. For each compound, five ethyl acetate solutions with concentrations in the range of interest were injected into the GC in triplicate. Linear regression analyses were conducted and the resultant response factors examined by a one-factor analysis of variance (ANOVA).

Replicate samples of control sheep plasma were fortified with the analytes to evaluate method bias and repeatability. Thirty microliters of a methanol solution (high concentration solution) containing camphor, 1,8-cineole, and *p*-cymene (99.3, 97.8, and 92.8 µg/mL, respectively) was delivered to 3.00 mL of plasma to produce high-fortification-level samples with concentrations of 0.993, 0.977, and 0.927 µg/mL for camphor, 1,8-cineole, and *p*-cymene, respectively. Eight replicate fortifications were prepared and analyzed by SPE–GC–FID. All plasma samples were also fortified with a 134-µg/mL terpinolene solution immediately prior to SPE extraction. Similarly, 30 µL of a low concentration solution in methanol (camphor at 19.9 µg/mL, 1,8-cineole at 19.6 µg/mL, and *p*-cymene at 18.6 µg/mL) was delivered to 3.00 mL of plasma to produce low-fortification-level samples with concentrations of 0.199, 0.196, and 0.186 µg/mL for camphor, 1,8-cineole, and *p*-cymene, respectively. Eight replicate fortified samples were also prepared at this lower concentration level and analyzed. Additionally, seven control samples were fortified with terpinolene only and subjected to the method procedures.

Quantitative analyses were conducted using two methods. First, single-point external standard calibrations were used by comparing detector responses of the analytes from the sample extracts to responses from a standard solution containing 1.01 µg/mL camphor, 0.909 µg/mL 1,8-cineole, and 0.972 µg/mL *p*-cymene in ethyl acetate. Quantitation was also achieved by fortifying all samples with a known quantity of terpinolene. The concentration of terpinolene was 1.34 µg/mL of plasma, and the detector response attained from chromatographic analysis was assumed to be the response produced by any monoterpene with a plasma concentration of 1.34 µg/mL. A three-factor ANOVA was performed to determine if analyte recovery was impacted by any of the factors manipulated in the evaluation of bias and repeatability. These factors were: the analyte (camphor, 1,8-cineole, and *p*-cymene), the fortification level (high or low), the quantitation method (external standard or direct surrogate standard), and all two- and three-way interactions.

Method limits of detection (MLOD) were determined by fortifying three replicate control plasma samples with the analytes at the following concentrations: 0.249 µg/mL camphor, 0.245 µg/mL 1,8-cineole, and 0.233 µg/mL *p*-cymene. The samples were extracted according to the mentioned procedures, and the chromatographic responses were evaluated. The MLOD was defined as the concentration of analyte required to produce a detector response equal to three times the baseline noise (measured peak-to-peak). The method limit of quantitation (MLOQ) was similarly defined for each analyte as the concentration producing a detector response equal to ten times the baseline noise.

To assess the storage stability of plasma samples and plasma extracts, 16 replicate samples of sheep plasma (3.00 mL) were fortified with each analyte at the same high-level fortification level used to assess method bias and repeatability. Six control plasma samples (no analytes added) were also prepared. All plasma samples were also fortified with the surrogate standard at a concentration of 1.34 µg/mL. Eight of the fortified samples and three of the control samples were stored in 25-mL screw-cap culture tubes. The remaining samples (8 fortified and 3 control) were subjected to the SPE extraction procedure, except that the columns were not eluted. The fortified plasma samples and SPE columns were maintained at –12°C for 35 days. After that period, the plasma samples were subjected to the SPE–GC–FID procedures described in this work. The SPE columns were eluted with 1.00 mL ethyl acetate and the extracts subjected to chromatographic analyses.

Analyte concentrations in the storage stability samples were quantitated by both external standard calibration and direct quantitation versus the surrogate standard. A three-factor ANOVA was performed to determine if analyte recovery was impacted by any of the factors manipulated in the storage stability experiment. These factors were the analytes (camphor, 1,8-cineole, and *p*-cymene), the quantitation method (external standard or direct surrogate standard), the storage method (fresh plasma, stored plasma, or stored SPE column), and all two- and three-way interactions.

An artifact of storage was observed in the chromatograms of extracts derived from the stored SPE columns. These extracts were subjected to GC analyses employing mass selective detection. The chromatographic parameters were the same as reported for the GC–FID analyses, except that the detector

temperature was 280°C (transfer line) and the mass detector scanned *m/z* from 33 to 300 mass units.

To demonstrate that this method was suitable for pharmacokinetic studies, a lamb was administered camphor intravenously, and blood was drawn for analysis. For this evaluation, 3.15 g camphor was dissolved in 3.00 mL methanol and then thoroughly mixed with 75 mL of a 20% intravenous fat emulsion (Intralipid). The solution was delivered at 1 mL/min per 1 kg body weight into the jugular vein via an indwelling catheter using a variable flow peristaltic pump. Blood samples were drawn from the jugular vein from an indwelling catheter into 10-mL heparinized tubes. Plasma was separated by centrifugation and stored at –18°C prior to analysis for camphor.

Results and Discussion

Detector response data indicated that the three analytes, as well as the surrogate compound, yielded linear responses over the range of interest (Table I). Furthermore, detector response was proportional to concentration for each compound, as indicated by the inability to distinguish their intercepts from zero ($\alpha = 0.05$). Inspection of the response factors further demonstrated that a proportional response was produced over the ranges of interest (no relative standard deviation was greater than 3.5%, see Table I). These data indicated that single-point calibrations could be used for quantitative analyses.

Table I. Detector Response Data for Monoterpene Analytes and Surrogate Standard*

Terpene	Range (µg/mL)	R^2	Slope	*p*-value	$R_F \times 10^{-4}$ (RSD)
p-Cymene	0.25–5.2	0.9995	3690	0.968	2.70 (2.34%)
1,8-Cineole	0.23–4.9	0.9995	3070	0.867	3.25 (2.34%)
Camphor	0.25–5.5	0.9994	3060	0.827	3.28 (1.90%)
Terpinolene	0.23–5.0	0.9994	3490	0.239	2.78 (3.48%)

* R^2 is the coefficient of determination; *p*-value is the probability associated with testing the hypothesis that the *y*-intercept of the line is zero; and R_F is the mean response factor determined for the analyte over the range tested with relative standard deviation.

The bias and repeatability evaluation demonstrated that the SPE–GC–FID methodology provided excellent recovery and reproducibility for all terpenes at both fortification levels (Table II). Furthermore, the analytes were not detected in the control plasma samples. ANOVA results indicate that although the three-way interaction ($p = 0.918$) and the terpene × fortification level interaction ($p = 0.911$) were not significant, both the terpene × quantitative method ($p < 0.001$) and fortification level × quantitative method ($p < 0.0001$) interactions were significant.

These results indicate that analyte recovery was constant for each terpene at both fortification levels. Furthermore, the statistical analysis demonstrated that quantitation versus the surrogate yielded higher recoveries for some analytes. Inspection of the data indicates that, in particular, *p*-cymene recovery was overstated when using the surrogate standard directly for quantitation. The surrogate standard method also overstated recovery at the low fortification level for each analyte. Nonetheless, the precision afforded by the surrogate standard method was comparable to the external standard method, particularly at the higher fortification level. When samples are extracted in the field to be analyzed at another location at a later time, it may be advantageous to employ the surrogate standard method.

The final volume of the SPE extract was assumed to be 1.00 mL (volume of ethyl acetate used for analyte elution) for quantification purposes. This is contrary to common practice, which involves elution of the SPE column with a larger volume than used here, collecting as much of the extract as possible, assuming that all of the analyte was eluted from the column, and bringing the extract to a known volume. This typical approach was not desirable because 2.00 mL of ethyl acetate constituted a source of dilution that was determined to be unacceptable (a 4:1 solvent-to-sorbent ratio is recommended for SPE methods). The data obtained from method evaluation indicated that no overall compromise in accuracy or precision was introduced by this assumption.

One of the objectives of this work was to test the *a priori* hypothesis that direct quantitation versus a surrogate standard would yield similar results to the external standard method of quantitation. This hypothesis was based on two important predictions. First, analyte recoveries from the matrix were predicted to be very similar among the analytes because they are similar in structure and chemical properties. Second, the detector response factors were predicted to be identical among these compounds because FID response is proportional to the number of carbon atoms in the molecule (11). Evaluation of

Table II. Bias and Repeatability Results: Analyte Recovery of Terpenes from Sheep Plasma at Two Fortification Levels*

	Mean analyte recovery (SD)		
Terpene	Fortification	vs. External standard	vs. Surrogate standard
p-Cymene	0.927 µg/mL	95.0% (3.40)	112% (4.59)
p-Cymene	0.186 mg/mL	92.9% (5.12)	127% (15.9)
1,8-Cineole	0.977 µg/mL	98.2% (3.67)	97.7% (7.59)
1,8-Cineole	0.196 µg/mL	99.4% 5.39)	112% (15.7)
Camphor	0.993 µg/mL	104% (3.25)	101% (5.05)
Camphor	0.199 µg/mL	104% (5.93)	118% (15.1)

* Recovery values determined versus external standard calibration and direct comparison to a surrogate standard ($n = 8$).

the detector response data indicated that, although these compounds with identical numbers of carbon yield "similar" responses (response factors varied by about 20%; Table I), the differences among them were significant ($p < 0.0001$). Similarly, the positive bias in *p*-cymene recovery observed from quantitation versus the surrogate standard indicates that the efficiency of extracting these two compounds from plasma also differs significantly.

From a practical standpoint, the assumption that related compounds with identical carbon numbers yield identical response factors is valid. Excellent methods employing internal standards for quantitation by FID rely primarily on this assumption and yield excellent results. Likewise, other valuable methods that employ surrogate standards as a means to account for variable analyte recoveries rely on the assumption that chemically similar compounds yield similar recoveries. In the practical sense, this assumption is also valid. However, the results obtained in the evaluation of the method described here were evaluated statistically, not practically. The excellent precision obtained from both GC-FID analyses and the extraction method makes even minor deviations from the assumptions appear to be significant. In fact, either quantitation approach would yield excellent data for use in a pharmacokinetic study.

The SPE–GC–FID method yielded excellent detectability and sufficiently low limits of quantitation. The MLODs determined for the analytes were 0.236, 0.244, and 0.210 µg/mL for camphor, 1,8-cineole, and *p*-cymene, respectively. The MLOQs were 0.786, 0.813, and 0.700 µg/mL for these analytes (in the same order). It is highly likely that these limits could be significantly lowered by extracting larger volumes of plasma. These limits were not approached when a plasma sample collected from a sheep 6 h after camphor administration was subjected to these procedures (Figure 1).

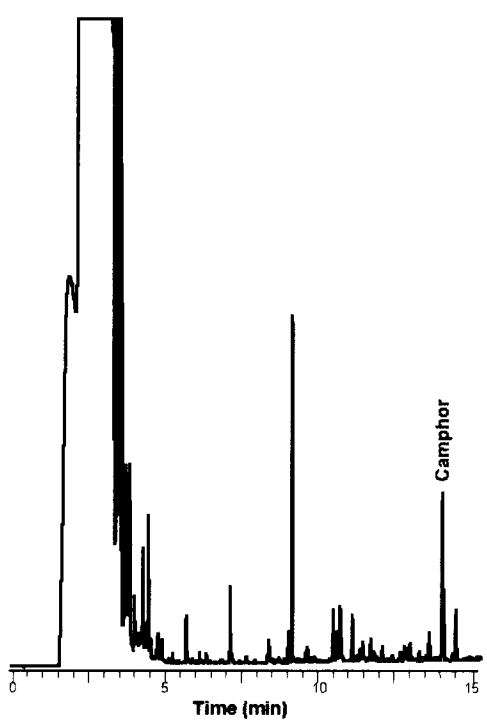

Figure 1. A chromatogram from the analysis of a plasma sample obtained from a sheep administered an intravenous dose of camphor. The plasma sample was collected 6 h after the camphor was delivered and subjected to the SPE–GC–FID method, except that the surrogate standard was not included. The camphor concentration was determined to be 1.48 µg/mL by comparison to the external standard.

Previous studies have indicated that analyte stability on stored SPE columns is variable and often a function of the stationary phase or storage conditions (or both) (12–14). The storage stability data indicate that plasma samples may be extracted with SPE columns and the columns stored for at least five weeks prior to chromatographic analysis (Table III). This is a nice feature, allowing for sample extraction and analysis to be separated not only spatially (extraction in a remote location) but also temporally (SPE columns may be shipped to a central lab and held for several weeks prior to analysis).

Statistical analysis indicated that there was no three-way interaction in the storage data ($p = 0.999$), but all possible two-way interactions were significant. The terpene × quantitative method interaction ($p < 0.0001$) suggests that the surrogate method yielded a higher recovery for *p*-cymene (as in the analysis of the bias and repeatability data). The terpene × storage method interaction ($p = 0.0013$) indicates that camphor and 1,8-cineole recoveries were elevated in stored plasma

Table III. Evaluation of Sample Storage Stability for Samples Fortified at the High Fortification Level*

	Mean analyte recovery (SD)		
Terpene	Storage Method vs.	External standard vs.	Surrogate standard
p-Cymene	Fresh	95.0% (3.40)	112% (4.59)
p-Cymene	Plasma	94.3% (9.48)	116% (6.77)
p-Cymene	SPE	90.8% (8.50)	121% (6.13)
1,8-Cineole	Fresh	98.2% (3.67)	97.7% (7.59)
1,8-Cineole	Plasma	106% (11.0)	110% (6.64)
1,8-Cineole	SPE	95.0% (9.69)	107% (6.24)
Camphor	Fresh	104% (3.25)	101% (5.05)
Camphor	Plasma	119% (12.4)	121% (6.81)
Camphor	SPE	104% (11.4)	115% (7.14)

* Recovery values determined versus external standard calibration and direct comparison to a surrogate standard ($n = 8$). "Fresh" plasma data corresponds to results obtained from extracting freshly fortified plasma (method bias and repeatability experiment; Table I). "Plasma" indicates that the fortified plasma was stored for 35 days prior to analysis. "SPE" indicates the SPE column was stored for 35 days prior to final elution of analytes.

versus fresh plasma and stored SPEs. Finally, the surrogate method was shown to yield high recovery values, particularly with the stored SPE method, as indicated by the interaction of quantitative method and storage method ($p = 0.0002$). Storage stability data suggest that analyzing freshly extracted plasma is not required. Excellent data may be obtained for all analytes even when the SPE column is stored for up to 35 days.

A chromatographic artifact was noted in the extracts eluted from stored SPE columns (Figure 2). Analysis of these extracts by GC–mass selective detection and subsequent injection of a known standard led to the identification of this compound as phenol. It is not clear why this phenol response (later to be identified in other extracts at much lower magnitudes) is so prevalent in extracts eluted from stored SPE columns. An experiment with stored SPE columns that had been conditioned only (no plasma extraction) and stored under identical conditions produced no such peak response. The presence of phenol, while interesting, does not interfere with this method.

Overall, the data obtained from the validation of this method indicate that camphor, 1,8-cineole, and *p*-cymene can be reliably extracted from sheep plasma and quantitated at low residue levels. Although quantitation can be easily achieved versus an introduced surrogate standard, less bias is introduced in the determination when the analytes are quantitated versus external standards. Furthermore, although recovery of two analytes was impacted by storing the plasma sample, storing the SPE column had no negative impacts on accuracy or precision. This method was demonstrated to be suitable for use in the conduct of a pharmacokinetic study of sagebrush monoterpenes.

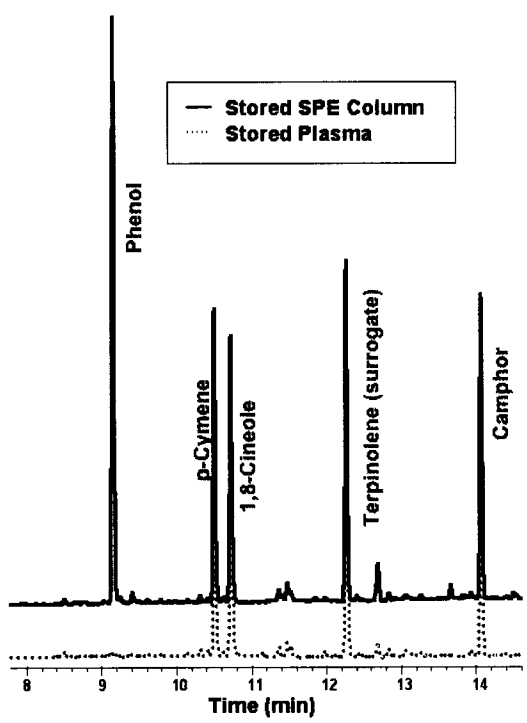

Figure 2. A chromatogram indicating the presence of phenol in extracts obtained from stored SPE columns.

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