Development and Validation of an Analytical Method for Quantitation of Alpha-Pinene Oxide in Rodent Blood and Mammary Tissue by GC-MS

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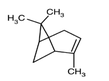
Abstract: 3122

Abstract



Alpha-pinene (AP)

Alpha-pinene Oxide (APO)

Alpha-pinene (AP), produced by pine trees and other plants, is the main component of turpentine and is used as a fragrance and flavor ingredient. Exposure to AP occurs via use of personal care and household cleaning products and in lumber industry. Despite widespread exposure, toxicity data for AP are limited. Alpha pinene oxide (APO) is a potential metabolite of AP in rodents and humans. Given its potential reactivity in tissues, establishing the extent of its formation and concentration in blood and tissues is an important component of the evaluation of the toxicity of AP. The objective of this work was to validate a method to quantitate APO in rat and mouse blood and mammary tissue, a potential target, in support of the toxicokinetic and toxicology studies. Standards were prepared by adding 5 µL of APO spiking solution to 100 µL rat blood followed by 300 µL ethyl acetate containing internal standard (IS, (+) limonene oxide). Sample was vortexed for 3 min and centrifuged for 3 min. The supernatant was analyzed by GC-MS in EI mode. The ions monitored were m/z 109 (APO) and 94 (IS). The method was successfully validated in male Sprague Dawley rat blood over the concentration range of 5.00 to 250 ng/mL. Matrix standard curves were linear (r ≥ 0.99), and accuracy measured as the percent relative error (%RE) values were ≤ ±15% for standards at all levels. Intra- and inter-day precision (% relative standard deviation, RSD) and accuracy (%RE) were ≤ 6.3% and ≤ ±5.4%, respectively. The limit of detection determined from the SD of the lower limit of quantitation (5 ng/mL), was 1.07 ng/mL. Absolute recoveries were 100-114% across all standard levels. Standards as high as 25000 ng/mL could be analyzed with 1000x dilution with %RE ≤ ±7.1% and %RSD ≤4.5%. APO was stable in rat blood for at least 70 days in frozen storage (-80 °C). The method was evaluated in male and female Harlan Sprague Dawley rat blood and B6C3F1 mouse blood: %RE values were ≤ ±5.3% and %RSD ≤7.8%. The method was also evaluated in female B6C3F1 and SD rat mammary tissue. Approximately 50 mg mammary tissue was homogenized with a 950 µL of water. APO was added to give concentration in the range 25-500 ng/g mammary, 100 µL of homogenate was extracted with ethyl acetate and analyzed as described above for blood. Mean %RE values were ≤ ±14.6% and %RSD ≤8.1%. These results demonstrate that the method is suitable for the analysis of APO in rodent blood and mammary tissues generated from toxicokinetic and toxicology studies.

Objective

To develop and validate an analytical method for the determination of APO in male Sprague Dawley (SD) rat blood (primary matrix) and in male and female Harlan Sprague Dawley rat and B6C3F1 mouse blood (secondary matrices) over a range of 5 to 250 ng/mL, and to partially validate in B6C3F1 mouse and SD rat mammary tissue homogenate over a range of 50 to 500 ng/mL.

Materials & Methods

Materials

Alpha-pinene oxide (APO; CAS No. 1686-14-2) and (+)-Limonene Oxide (LO; Internal Standard IS):Sigma Aldrich (Saint Louis, MO). Sprague Dawley (SD) and Harlan Sprague Dawley (HSD) rat blood; B6C3F1 mouse blood: BioIVT (Westbury, NY).

Sample Preparation

Standards and QC standards were prepared by spiking 100 µL blood with 5 µL APO spiking solution and extracted with 300 µL internal standard solution (IS; 66.7 ng/mL) consisting of (+)-limonene oxide in ethyl acetate. Each tube was vortex extracted for 3 minutes, and centrifuged at room temperature at 16000*g* for 3 minutes. Supernatant was transferred to a clean glass vial with a micro-insert.

Preparation of Mammary Homogenate

Mammary homogenate was prepared by adding 50-mg aliquots of mammary tissue in 2-mL plastic homogenization tubes containing 2.8 mm stainless steel grinding balls and ~ 950 µL of water (1:19, tissue:water). The tubes were placed in a Genogrinder (Spex Sample Prep, Metuchen, NJ) for five 1-minute cycles of 1750 RPM. A 100-µL aliquot of each homogenate was transferred to a 1.5-mL microcentrifuge tube, and extracted as described above.

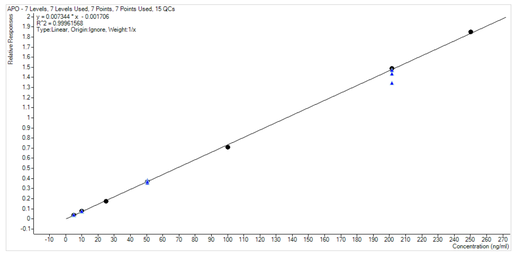
GC-MS System; Software	Agilent 7890A GC / 5975 MSD; Masshunter B.07.00 SP2 (Agilent Technologies, Santa Clara, CA)
Autosampler Vial Size	1.5 mL
Syringe Vol.	10 µL
Sample Temp	Room Temp
Sample Volume	1 µL
Column	Agilent DB-5 (30 m x 0.25 mm ID, 0.25-µm film)
Carrier Gas	Helium at 1.2 mL/min.
Oven Temp. Program	40 °C for 5 min, ramp to 140 °C at 5 °C/min, hold 20 min, ramp to 300 °C at 20 °C/min, total time = 38 min.
Retention Time	~16.5 min APO, ~17.5 min LO
Injector Temp.; Injection Mode	200 °C; Splitless
Auxiliary Temp.; MS Source Temp.	280 °C; 230 °C
Quad Temp.; MS Ionization Mode	150 °C; Electron Ionization (70 eV)
Acquisition Mode	Single ion monitoring (SIM); *m/z* 109 (APO) and 94 (IS)

Validation Design

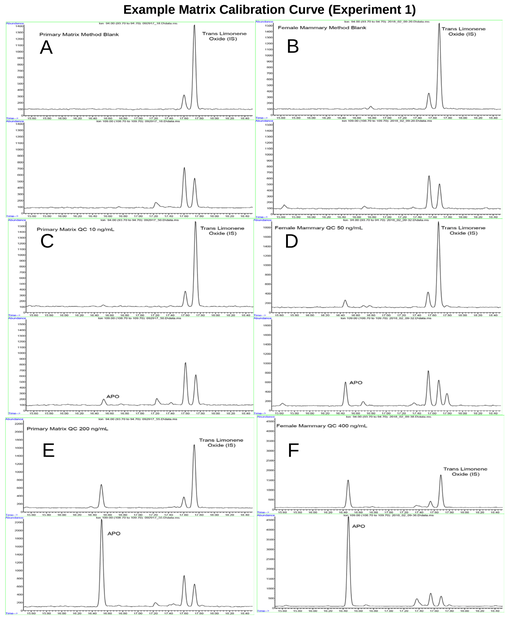
Linearity: 7-point calibration curve in male SD rat blood over the range 5-250 ng/mL on each of 3 days
Recovery: Compare a set of matrix standards to equivalent set of solvent standards
Selectivity: 6 method blanks (with IS) and 6 matrix blanks (without IS)
Sensitivity: 6 replicates at the lowest concentration level to define LLOQ and LOD
Intra- and Inter-Day Precision & Accuracy: Triplicate matrix standards at 3 levels on each of 3 days. Precision calculated as %RSD; Accuracy calculated as Relative Error (RE)
Instrument Drift: Matrix standards run at start and end with multiple samples in between
Carryover: 3 method blanks after high matrix standard
Method Extension: Triplicate matrix standards at 25000 ng/mL
Autosampler Stability: Triplicate matrix standards at 2 levels; stored on autosampler overnight
Frozen Matrix Stability: Triplicate matrix standards at 2 levels; stored at -80 °C up to > 60 d
Secondary Matrix Evaluation: 6 method blanks, 6 matrix blanks, and 6 replicates at 2 x LLOQ in each secondary matrix; quantitated using primary matrix curve (male SD rat blood)
Partial Validation of Mammary Tissue: Selectivity: 3 method blanks (with IS) and 3 matrix blanks (without IS); Precision and Accuracy: Triplicate matrix standards at 2 levels prepared in mammary homogenate and quantitated using the primary matrix curve (SD rat blood over the range of 50-500 ng/mL)

Results – Method Validation

The method was successfully validated over the concentration range 5-250 ng/mL in male SD rat blood (Primary Matrix). The LOD, determined from the standard deviation at the LLOQ (5 ng/mL), was 1.07 ng/mL. A partial validation for the analysis of mammary tissue homogenate was performed, for the analysis of mammary tissue the concentration range was 25-500 ng/mL.



Example Matrix Calibration Curve (Experiment 1)



Representative Extracted Ion Chromatograms of IS, *m/z* 94 (top) and APO, *m/z* 109 (bottom) in Primary Matrix, Male SD Rat Blood (Left) and Female Rat Mammary Homogenate (Right)
A: Method Blank in Primary Matrix, B: Method Blank in Female Mammary Homogenate, C: Primary Matrix QC 10 ng/mL, D: Female Mammary Homogenate QC 50 ng/mL, E: Primary Matrix QC 200 ng/mL, F: Female Mammary Homogenate QC 400 ng/mL

Results (cont'd)

Recovery in Blood			
Nominal Conc. (ng/mL)	**Matrix Standard Response**	**Solvent Standard Response**	**Absolute Recovery[a] (%)**
5.00	125.75	125.24	100
10.1	295.51	274.78	108
25.0	652.48	571.46	114
50.3	1292.77	1252.40	103
100	2533.72	2336.75	108
201	5200.40	4667.65	111
250	6474.17	6003.91	108
		Mean Recovery =	**108**
		Variation[b] =	**6.6**

Solvent standards prepared same as matrix standards, except water used instead of blood.
[a] Absolute Recovery = (Matrix Standard Response / Solvent Standard Response) x 100
[b] Variation = Highest % Recovery – Lowest % Recovery

- Absolute recovery ± 14% at all concentrations.

	Intra-Day Precision & Accuracy[a]		Inter-Day Precision & Accuracy[b]	
Nominal Conc. (ng/mL)	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean RE (%)**	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean RE (%)**
10.1	10.6 (2.0%)	5.4	10.6 (3.7%)	-4.9
50.3	50.3 (3.5%)	-0.1	50.1 (5.9%)	-0.4
201	194 (4.7%)	-3.9	204 (6.3%)	1.3
25000 (1000 -Fold Dilution)	26806 (4.5%)	7.1	N/A	

[a] n = 3 (within calibration curve no. 1)
[b] n = 9 (across calibration curves nos. 1, 2, and 3)

- Intra- and inter-day precision and accuracy ≤ ±6% and ≤ ±5%, respectively, for QC standards prepared at 10.1, 50.3, and 201 ng/mL.
- Standards as high as 25000 ng/mL could be analyzed by diluting 1000-fold into the validated range, with %RE = 7.1% and %RSD = 4.5%.

Stability in Blood			
Stability Condition	**Nominal Conc. (ng/mL)**	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean % of Day 0[a]**
Autosampler, 5 days	10.1 50.3 201	10.6 (6.2%) 55.2 (11.0%) 206 (5.1%)	99.5 116 99.0
-80 °C, 15 days	10.1 50.3 201	10.4 (5.5%) 41.9 (2.3%) 170 (3.2%)	97.8 88.2 81.7
-80 °C, 27 days	10.1 50.3 201	9.86 (10.2%) 44.5 (3.0%) 176 (0.9%)	93.1 93.6 84.4
-80 °C, 70 days[b]	10.1 50.3 201	10.4 (2.2%) 50.3 (1.7%) 190.5 (2.9%)	98.1 106 91.7

[a] % of Day 0 = (Found Stored Conc. / Found Day 0 Conc.) x 100
[b] Interim points were assessed at 15 and 27 days

- Autosampler stability for at least 5 days Mean % of Day 0 = 98.7-116% and %RSD ≤ 11.0%
- Frozen matrix stability for up to 70 days Mean % of Day 0 = 81.7-106% and %RSD ≤ 10.2%

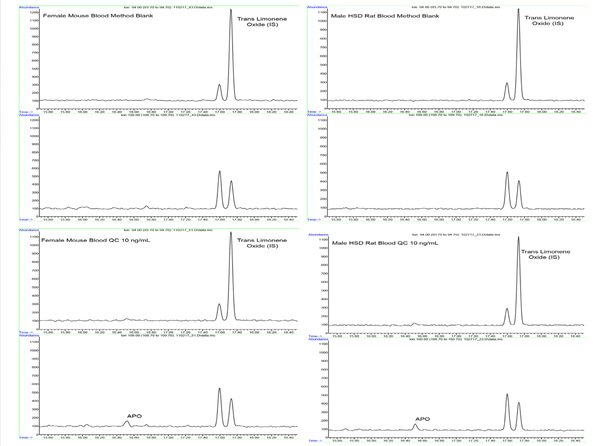
Secondary Matrix Evaluations: Precision & Accuracy (n = 6)			
Matrix	**Nominal Conc. (ng/mL)**	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean RE (%)**
Male HSD rat blood	9.73	9.87 (7.8%)	-1.3
Female HSD rat blood	9.73	9.77 (6.0%)	-2.3
Male B6C3F1 mouse blood	9.73	9.64 (6.6%)	-0.9
Female B6C3F1 mouse blood	9.73	10.3 (4.6%)	5.3

- The method was evaluated for male and female HSD rat blood and B6C3F1 mouse blood; %RE values were ≤ ±6% and %RSD ≤ 8%.

Partial Validation: Precision & Accuracy (n = 3)			
Matrix	**Nominal Conc. (ng/mL)**	**Mean Found Conc. (ng/mL) (%RSD)**	**Mean RE (%)**
Female SD rat mammary	49.9 411	43.5 (0.39%) 374 (7.9%)	12.9 8.9
Female B6C3F1 mouse mammary	49.9 411	42.6 (8.1%) 385 (7.6%)	14.6 6.3

- Partial validation in female rat and mouse mammary homogenate was evaluated; %RE values were ≤ ±15% and %RSD ≤ 9%.

Results (cont'd)



Representative Extracted Ion Chromatograms of IS, *m/z* 94 (top) and APO, *m/z* 109 (bottom) in Male HSD Rat Blood (right) and Male B6C3F1 Mouse Blood (left) [Secondary Matrix Evaluation]

Method Validation Summary

Validation Parameter	Acceptance Criteria	Results
Linearity	*r* ≥ 0.99 and %RE ≤ ±15% (≤ ±20% at LLOQ)	**Passed**: *r* ≥ 0.99 and %RE ≤ ±13.7% for all calibration standards
Recovery	Extraction recovery > 80% at each level, with variation ≤ 20% across the levels	**Passed**: Extraction recovery 100-114% at all levels.
Selectivity	Method blanks ≤ 30% of LLOQ response	**Passed**: Mean method blank response ≤ 19.0% of LLOQ
Sensitivity (LLOQ and LOD)	LLOQ: %RE ≤ 20% and %RSD ≤ ±20%; LOD = 3xSD for LLOQ replicates	**Passed**: %RE ≤ ±18.3% and %RSD ≤ 6.4% at **5 ng/mL (LLOQ) LOD = 1.07 ng/mL**
Intra- and Inter-day Precision & Accuracy	Mean %RE ≤ ±15% and %RSD ≤ 15%	**Passed**: Mean %RE ≤ ±5.4% and %RSD ≤ 4.7% (Intra-day) ; Mean %RE ≤ ±4.9% and %RSD ≤ 6.3% (Inter-day)
Method Extension	Mean %RE ≤ ±20% and %RSD ≤ 20%	**Passed**: Mean %RE = -1.1% and %RSD = 5.8%
Autosampler Stability	Mean % of Day 0 = 100 ±20% and %RSD ≤ 20%	**Stable**: Mean % of Day 0 = 99.0 - 116% and %RSD ≤ 11.0%
Frozen Matrix Stability	Mean % of Day 0 = 100 ±20% and %RSD ≤ 20%	**Stable (70 Days)**: Mean % of Day 0 = 81.7-106% and %RSD ≤ 10.2%
Secondary Matrix Evaluations - Male, female HSD blood - Male, female B6C3F1 blood	Mean %RE ≤ ±15% and %RSD ≤ 15%; Method blanks ≤ 30% of LLOQ response	**Passed**: Mean %RE ≤ ±5.3% and %RSD ≤ 7.8% **Passed:** Mean method blank response = 3.4%
Partial Validation Evaluations - Male, female SD rat mammary tissue - Male, female B6C3F1 mouse mammary tissue	Mean %RE ≤ ±15% and %RSD ≤ 15%; Method blanks ≤ 30% of LLOQ response	**Passed**: Mean %RE ≤ ±14.6% and %RSD ≤ 8.1% **Passed:** Mean method blank responses ≤ 1.63%

Conclusions

Alpha-pinene Oxide (APO) can be quantitated in male and female rat and mouse blood using this solvent extraction and GC-MS method.

The method was successfully validated over the range 5-250 ng/mL in whole blood. Validation parameters included linearity, recovery, selectivity, sensitivity, precision, accuracy, and stability. It was also demonstrated that blood concentrations as high as 25000 ng/mL could be analyzed after dilution into the validated concentration range.

The method was partially validated in mammary tissue homogenate. The partially validated parameters included selectivity, precision, and accuracy.

These methods are currently being applied for the analysis of APO in rodent blood and mammary tissue samples from toxicokinetic and toxicology studies.

Acknowledgement

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