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DETERMINATION OF (*E*)-1,2,3,4-TETRAHYDRO-1,1,4,4-TETRAMETHYL-6-(1-METHYL-2-PHENYLETHENYL)NAPHTHALENE, AN ANTIACNE AGENT, AND ITS PHENOLIC METABOLITE IN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of (*E*)-1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-(1-methyl-2-phenylethenyl)naphthalene (I) and its phenolic metabolite, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-methylethenyl]phenol (II) in plasma. The assay for both compounds involves precipitation of the plasma proteins with acetonitrile, followed by extraction of the entire mixture into methyl *tert.*-butyl ether and subsequent analysis by reversed-phase HPLC. The overall recovery of I and II was 96.9 ± 5.3 and 96.2 ± 5.8% for dog plasma, 80.0 ± 4.0 and 93.5 ± 5.0% for human plasma and 97.4 ± 2.9 and 97.6 ± 9.6% for rat plasma, respectively. The sensitivity limit is 20 ng/ml of plasma for both compounds I and II using UV detection at 280 nm. The HPLC assay was used in studies in the dog and in the rat.

INTRODUCTION

Compound I, (*E*)-1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-(1-methyl-2-phenylethenyl)naphthalene (Fig. 1), synthesized by Loeliger et al. [1] is a member of a new class of highly active retinoids termed arotinoids. It has shown high antipapilloma activity [1] and is currently under development as an antiacne agent [2,3].

Studies on the in vitro biotransformation of I using 9000 *g* supernatant of incubated rat liver prepared from phenobarbital pretreated rats [4] indicated that the major metabolite was the *p*-hydroxy phenolic analogue II, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-methylethenyl]phenol (Fig. 1).

A rapid, sensitive and selective high-performance liquid chromatographic

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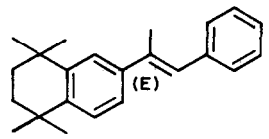

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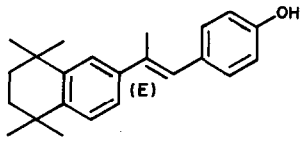

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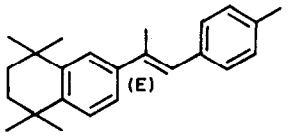
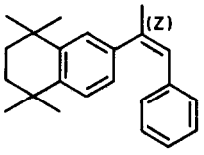


III



IV

Fig. 1. Chemical structures for the compounds referred to in the text.

(HPLC) assay was developed for the determination of compounds I and II in plasma. The method presented herein determines compounds I and II by reversed-phase HPLC using their ultraviolet (UV) absorbance at 280 nm for quantitation. The methyl analogue, (*E*)-1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-[1-methyl-2-(4-methylphenyl)ethenyl]naphthalene (III, Fig. 1), is used as the internal standard.

Compound IV (Fig. 1), the *Z* isomer of compound I was investigated as a potential contaminant and found not to be present.

The stability of I was demonstrated in dog, human and rat plasma at ambient temperature for 24 h and upon storage at −17°C for 60–70 days. Long-term stability studies for storage at −17°C and −70°C for up to six months are in progress.

The HPLC assay was used to monitor plasma concentrations of I and II in toxicological range finding studies in the dog and rat following the administration of single ascending oral doses of compound I. It will also be applied to monitor ongoing biopharmaceutic and pharmacokinetic studies.

EXPERIMENTAL

Analytical standards

Compound I ($C_{23}H_{28}$, MW 304.5, m.p. 81–82°C), compound II ($C_{23}H_{28}O$, MW 320.5, m.p. 138–139°C), compound III ($C_{24}H_{30}$, MW 318.5, m.p. 84–85°C) and compound IV (*Z* isomer of I ($C_{23}H_{28}$, MW 304.5, m.p. 43–45°C) were obtained from Hoffmann-La Roche (Nutley, NJ, U.S.A.) and were of pharmaceutical grade purity (>99%) for use as the analytical standards.

Preparation of standard solutions

Prepare stock solutions of compounds I, II and III in separate 10-ml amberized volumetric flasks [5] by dissolving 10 mg of each compound into 2 ml of methanol. Sonicate if necessary for 5–10 min for complete solubilization and dilute to volume with methanol. These stock solutions (containing 1.0 mg/ml) are used to prepare seven mixed standard solutions by suitable dilutions in methanol to contain both compounds I and II in concentrations of 20, 50, 100, 200, 500, 1000 and 2000 ng per 50 μl; the standard solution 8 contains 200 ng per 50 μl of compound III.

Aliquots (50 μl) of solutions 1–7 and of solution 8, the internal standard, are diluted with 100 μl of methanol and used to prepare an authentic, external standard calibration curve to establish the linearity and reproducibility of the HPLC system.

Aliquots (50 μl) of solutions 1–7 and of solution 8, the internal standard, are added to separate 1.0-ml specimens of control plasma and processed along with the samples to establish a processed (recovered) standard calibration curve for direct quantitation of unknowns.

Calibration of compounds I and II by HPLC

Calibration (external standard) curves of the peak-height ratio of I and II to III versus concentration of compound injected are constructed. Fresh calibration curves of the external standards and of the processed (recovered) standards are prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system.

Reagents

Acetonitrile, methanol and methyl *tert.*-butyl ether, of analytical reagent grade (>99% purity), were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Instrumental parameters

A prepacked 25 cm×4.6 mm I.D. stainless-steel column containing 5-μ spherical Sepralyte™ microparticulate C_{18} bonded silica gel generating 19 000 plates/m (Analytichem International, Harbor City, CA, U.S.A.) was used at ambient temperature.

The HPLC system consisted of a Model M6000A reciprocating piston pump (Waters Assoc., Milford, MA, U.S.A.), a WISP Model 710B autoinjector and a

Spectroflow Model 757 absorbance detector (Kratos, Ramsey, NJ, U.S.A.). The isocratic mobile phase used was a mixture of methanol–acetonitrile–water (90:6:4) at a pressure of ca. 14 MPa (200 p.s.i.) and a constant flow-rate of 2 ml/min. The UV detector was operated at 280 nm at a sensitivity of $5 \cdot 10^{-3}$ a.u.f.s., and the chart speed on the 10-mV recorder, Model 7132A (Hewlett-Packard, Palo Alto, CA, U.S.A.) was 0.64 cm/min. The WISP autoinjector was programmed to run for 16 min (14 min run time + 2 min purge and rinse) per sample using methanol as the rinse solvent. Under these conditions 50 ng of I and II and 20 ng of III injected gave nearly full-scale pen response. The retention times (tR) of I, II and III were 6.9, 3.3 and 8.9 min, respectively; their corresponding capacity factors (k') were 3.9, 1.3 and 5.3, respectively. The minimum detectable amount of I and II was 2.0 ng injected equivalent to 20.0 ng/ml of plasma, which corresponds to a signal-to-noise ratio of approximately 4:1.

Analytical procedure

All glassware was amberized to prevent the possible light-induced isomerization of these compounds [5]. Into a glass-stoppered amberized 15-ml centrifuge tube, transfer 50 µl of solution 8 (equivalent to 200 ng of III, the internal standard), 50 µl of methanol and 1 ml of plasma. Mix for a few seconds at the highest speed setting of a vortex mixer (for human plasma only, add 0.5 ml of water and vortex again; see Discussion). Add 2 ml of acetonitrile and vortex again for 20 s. Extract the entire mixture with 10 ml of methyl *tert.*-butyl ether by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80–100 strokes/min. Centrifuge the samples in a refrigerated centrifuge (Model DPR-6000 Damon/IEC, Needham, MA, U.S.A.) at 5°C for 10 min at 2500–3000 rpm (ca. 2000 g). Transfer 9.0 ml of the upper organic layer into a 15-ml amberized centrifuge tube. Evaporate the extract to dryness at 50°C under a stream of clean dry nitrogen. Dissolve the residue in 200 µl of methanol and transfer the solution into a low-volume insert fitted on a spring of an amberized Waters glass injection vial capped with a self-seal septum cap (Waters, Part No. 73010). The autoinjector (WISP 710B) was programmed to inject 20 µl for HPLC analysis. Typical chromatograms of plasma extracts are shown in Fig. 2.

Recovered standard curve

Along with the samples, process eight 1.0-ml specimens of control plasma, one to be used as a control blank to which 100 µl of methanol are added and seven to be used for the preparation of the processed (recovered) standards to which 50 µl of solutions 1–7 (equivalent to 20, 50, 100, 200, 500 ng, 1 µg and 2 µg of I and II and 50 µl of solution 8 (equivalent to 200 ng of III) are added per ml of plasma. These standards are to be used to establish the processed (recovered) standard curve for the direct quantitation of the unknowns.

External standard curve

An external standard curve of the authentic compounds is prepared by combining 50-µl aliquots of solutions 1–7, 50 µl of solution 8 and 100 µl of methanol. A 20-µl aliquot (out of 200 µl) is injected to establish an external standard cali-

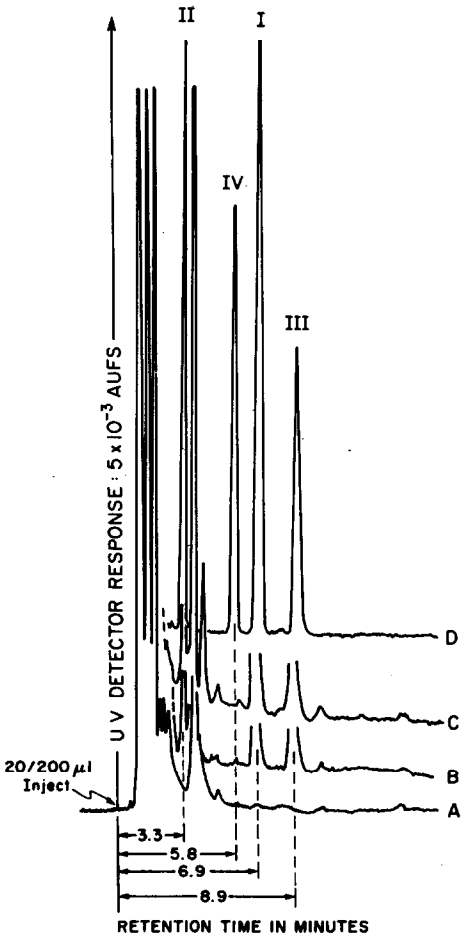


Fig. 2. Chromatograms of the HPLC analysis of methyl *tert.*-butyl ether extracts of (A) control dog plasma; (B) dog plasma following oral dosing of I at 400 mg/kg on day 1; (C) authentic standards of I, II and III recovered from control dog plasma; and (D) authentic standards of compounds I, II, III and IV (equivalent to 500 ng/ml I, II and IV and 200 ng/ml III).

bration curve to verify the linearity and reproducibility of the automated HPLC assay.

RESULTS AND DISCUSSION

A rapid, sensitive and selective HPLC assay was developed for the determination of compounds I and II from plasma using UV detection at 280 nm for quantitation. This method enabled the rapid and accurate quantitation of compounds I and II for routine analysis of the large number of samples obtained during preclinical pharmacokinetic and toxicological studies. Although compound I was shown to be stable under the sample preparation techniques used (Table III) amberized glassware was used for the development and validation of the method

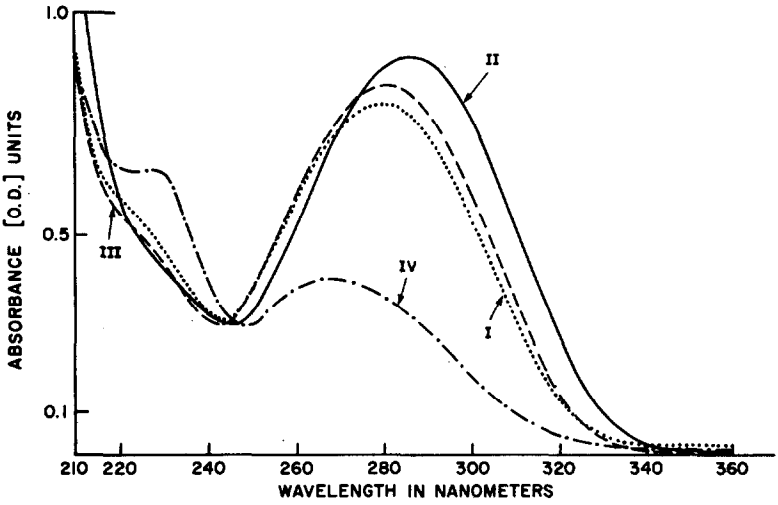


Fig. 3. UV absorption spectra of compounds I, II, III and IV in methanol.

as well as sample analysis to prevent the possible light-induced isomerization reported for these compounds [5]. Compound IV (50 ng injected; equivalent to 500 ng/ml), the *Z*-isomer of compound I, was found to be well resolved from the other compounds using the same chromatographic conditions (Fig. 2).

The major UV absorption bands occur at 280–286 nm for compounds I, II and III and at 229 and 268 nm for compound IV (see Fig. 3). The Kratos Model 757 absorbance detector operated at 280 nm or the Waters Model 440 absorbance detector used in conjunction with a 280-nm wavelength kit and a medium-pressure mercury lamp allowed for the quantitation of I, II, III and IV with comparable sensitivity.

Reversed-phase HPLC analysis is the method of choice, since it resolves compounds I, II, III and IV with optimum resolution, peak symmetry and sensitivity.

Due to the lower overall recovery of compounds I and II from human plasma, compared to dog and rat plasma, the addition of 0.5 ml of water to human plasma prior to acetonitrile precipitation resulted in acceptable recovery. This modification is recommended for human plasma only.

A Waters 5-μm C_{18} Nova-Pak Radial Pak cartridge attached to a Waters Z-Module was also investigated. The chromatographic behavior was identical to the Analytichem column, which was chosen for ease of operation and economy.

Compound III, the methyl analogue of I, was chosen as the internal standard in the assay, due to its similar extraction and chromatographic behavior as compounds I and II. Compound III has not been identified as a metabolite of compound I.

Calculations and assay validation

The concentrations of I and II in the unknowns were determined by interpolation from a least-squares regression equation [weighted ($1/y$) linear equation: $y=mx+b$] of the calibration data (processed by a Hewlett-Packard Model 3357B

TABLE I

STATISTICAL VALIDATION OF THE HPLC ASSAY IN HUMAN PLASMA FOR COMPOUND I

Concentration added (ng/ml)	Concentration found (ng/ml)	Coefficient of variation (%)
Intra-assay variability ($n=3$)		
20.0	17.4 ± 0.2	1.1
50.0	51.3 ± 1.3	2.6
100	104 ± 5.2	5.0
200	211 ± 11.6	5.5
500	522 ± 12.2	2.3
1000	997 ± 22.3	2.2
2000	1970 ± 37.9	1.9
Average		3.0
Inter-assay variability ($n=6$)		
20.0	20.3 ± 0.6	3.0
50.0	50.6 ± 1.2	2.4
100	98.8 ± 2.3	2.4
200	199 ± 5.0	2.5
500	505 ± 10.8	2.1
1000	974 ± 15.8	1.6
2000	2020 ± 15.9	0.8
Average		2.1

laboratory automation system) of the recovered standards processed along with the unknowns using peak-height ratios (peak height of compounds I or II to the peak height of the internal standard, III) versus concentration of I and II per ml of plasma. Typical calibration curves as defined by the equation $y=0.0061x+0.0072$ for I and $y=0.0068x-0.0020$ for II were linear from 0.02 to 2 μg of I and II per ml of plasma. The correlation coefficients (r) were 0.998 and 0.996 and the average deviation from the line was 6.1 and 5.7% for I and II, respectively. The mean intra- and inter-assay coefficients of variation (C.V.) in human plasma were 3.0 and 2.1% for I and 5.4 and 5.0% for II, respectively (Tables I and II). The mean intra- and inter-assay C.V. values in dog plasma were 2.5 and 1.6% for I and 3.0 and 2.1% for II, respectively. The mean intra- and inter-assay C.V. values in rat plasma were 6.0 and 2.4% for I and 9.4 and 3.6% for II, respectively.

The determination of percentage recovery is calculated (with each analytical experiment) by comparing the absolute response (peak height) of the processed (recovered) standards to the absolute response (peak height) of the external standards.

Percentage recovery and sensitivity limits

The overall recovery of I and II was 96.9 ± 5.3 and 96.2 ± 5.8% for dog plasma, 80.0 ± 4.0 and 93.5 ± 5.0% for human plasma and 97.4 ± 2.9 and 97.6 ± 9.6% for rat plasma, respectively. The sensitivity limit of the assay (which corresponds to

TABLE II

STATISTICAL VALIDATION OF THE HPLC ASSAY IN HUMAN PLASMA FOR COMPOUND II

Concentration added (ng/ml)	Concentration found (ng/ml)	Coefficient of variation (%)
Intra-assay variability ($n=3$)		
20.0	19.2 ± 0.6	3.0
50.0	48.7 ± 1.7	3.4
100	102 ± 6.7	6.5
200	212 ± 20.6	9.7
500	517 ± 35.6	6.9
1000	1000 ± 49.5	4.9
2000	1980 ± 68.2	3.5
Average		5.4
Inter-assay variability ($n=6$)		
20.0	21.0 ± 0.5	2.2
50.0	49.2 ± 3.5	7.2
100	96.5 ± 6.7	7.0
200	205 ± 7.4	3.6
500	496 ± 40.7	8.2
1000	998 ± 53.7	5.4
2000	2010 ± 28.5	1.4
Average		5.0

a signal-to-noise ratio of approximately 4:1) is 20.0 ng/ml of plasma for both compounds I and II using 1 ml of plasma.

Stability studies on compound I in human, dog and rat plasma

Ambient temperature. Compound I was added to fresh control human, dog and rat plasma and analyzed after remaining in clear glassware at ambient (24°C) temperature from 0 to 24 h. The mean changes observed were ±2.5, −4.5 and ±3.8% for human, dog and rat plasma, respectively (Table III). Compound I is stable under the sample preparation techniques described.

Long-term storage stability at −17°C and −70°C. In order to evaluate the storage stability of compound I at −17°C and −70°C for up to 180 days, fresh control human, dog and rat plasma was spiked with 500 ng ml/I. The spiked plasma was divided into aliquots and stored at −17°C and −70°C. The −17°C samples were analyzed in quintuplicate through days 60–70. The mean changes observed were ±1.9, ±3.0 and ±4.3% for human, dog and rat plasma, respectively (Table IV). The data indicate that compound I was stable throughout the storage interval at −17°C. Day 180 samples will be analyzed at the appropriate time. Since degradation was not observed at −17°C, the samples stored at −70°C will not be analyzed at this time.

TABLE III

STABILITY OF COMPOUND I IN HUMAN, DOG AND RAT PLASMA AT AMBIENT (24°C) TEMPERATURE

The amount of compound I added was 500 ng/ml; $n=3$ in all cases; the percentage change was as compared to 0 h.

Time (h)	Concentration found (ng/ml)					
	Human		Dog		Rat	
	Mean	Percentage change	Mean	Percentage change	Mean	Percentage change
0	458	-	478	-	495	-
1	465	+1.5	452	−5.4	497	+0.4
4	447	−2.4	450	−5.9	491	−0.8
24	442	−3.5	468	−2.1	546	+10.3
Mean		±2.5		−4.5		±3.8

Application of the method to biological specimens

The HPLC assay was used to determine the plasma concentrations of I and II in four-week studies in the dog and the rat following single 50, 100, 200 or 400 mg/kg per day oral doses of compound I. Plasma concentrations of I and II found on day 1 of a four-week study in dogs administered oral doses of 400 mg/kg I are tabulated in Table V. In most cases concentrations of I and II were measurable for 24 h.

TABLE IV

LONG-TERM STABILITY OF COMPOUND I IN HUMAN, DOG AND RAT PLASMA AT −17°C

The amount of compound I added was 500 ng/ml; $n=5$ in all cases; the percentage change was as compared to day 1; N.S. = no sample.

Time (h)	Concentration found (ng/ml)					
	Human		Dog		Rat	
	Mean	Percentage change	Mean	Percentage change	Mean	Percentage change
1	434	-	480	-	516	-
7	443	+2.1	N.S.	-	N.S.	-
30	N.S.	-	472	−1.7	535	+3.7
60–70	441	+1.6	500	+4.2	491	−4.8
Mean		+1.9		±3.0		±4.3

TABLE V

PLASMA CONCENTRATIONS OF COMPOUNDS I AND II ON DAY I OF A FOUR-WEEK STUDY IN DOGS ADMINISTERED ORAL DOSES OF 400 mg/kg I

N.M. = non-measurable (< 20 ng/ml).

Dog	Sex	Time (h)	Concentration (μg/ml)	
			I	II
923	Female	0	N.M.	N.M.
		0.5	N.M.	N.M.
		1	0.05	0.09
		2	0.19	0.44
		4	0.26	0.95
		7	0.12	0.97
		24	N.M.	0.78
924	Female	0	N.M.	N.M.
		0.5	N.M.	N.M.
		1	0.18	0.17
		2	0.42	0.77
		4	0.26	1.19
		7	0.12	0.88
		24	0.95	9.77
925	Male	0	N.M.	N.M.
		0.5	N.M.	N.M.
		1	0.08	0.06
		2	0.44	0.55
		4	0.37	1.17
		7	0.17	0.98
		24	0.43	5.66
926	Male	0	N.M.	N.M.
		0.5	N.M.	N.M.
		1	0.09	0.06
		2	0.39	0.36
		4	0.29	0.89
		7	0.14	0.78
		24	N.M.	0.48

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