Journal of Chromatography, 136 (1977) 391–399
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CHROM. 9943

DETERMINATION OF THE ANTI-INFLAMMATORY AGENT CARPROFEN, (D,L)-6-CHLORO-α-METHYLCARBAZOLE-2-ACETIC ACID, IN BLOOD BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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(Received December 10th, 1976)

SUMMARY

A rapid, sensitive, and specific high-pressure liquid chromatographic (HPLC) assay was developed for the determination of (D,L)-6-chloro-α-methylcarbazole-2-acetic acid (carprofen) in blood. The assay involves extraction into diethyl ether from blood buffered to pH 6. The overall recovery of carprofen from blood is 97.3 ± 5.3% (S.D.), and the sensitivity limit of detection is 100–200 ng/ml of blood using a UV detector at 254 nm or 3 ng/ml of blood using a fluorescence detector with excitation at 240 nm and emission at wavelengths greater than 350 nm. The HPLC assay is amenable to rapid routine analysis of clinical specimens, and the data obtained using this assay showed an excellent correlation coefficient (0.99) compared with a previously published spectrofluorometric assay. The method was used to monitor the blood level–time fall-off profiles in four subjects following single and multiple dose administration of carprofen.

INTRODUCTION

The compound (D,L)-6-chloro-α-methylcarbazole-2-acetic acid (carprofen, compound I in Fig. 1) was synthesized by Berger[1] and is a member of a series of carbazoles undergoing pharmacological testing as anti-inflammatory agents[2].

Previously published luminescence and electron-capture gas–liquid chromatographic (EC–GLC) procedures for compound I in blood and urine[3,4] were time-consuming for routine analysis of the large number of specimens usually obtained from clinical studies. Consequently, a sensitive and specific high-pressure liquid chromatographic (HPLC) assay was developed for the determination of compound I in blood. The HPLC assay was equivalent to the luminescence and EC–GLC procedures in sensitivity and specificity but was much simpler to use in routine analysis.

The analogous compound 2-[(D,L)-6-chloro-2-carbazolyl]-propanol (compound II in Fig. 1) was used as the reference standard in the assay.

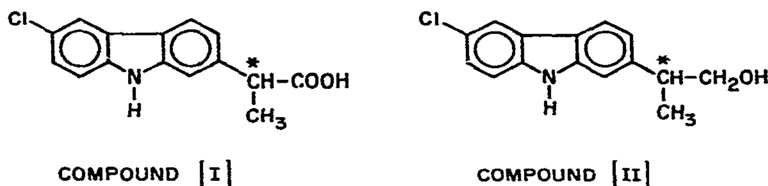

Fig. 1. Chemical structures of compounds I and II. The asterisk indicates the asymmetric carbon atom.

EXPERIMENTAL

HPLC analysis of compound I in blood

Column. The column used was a 0.25 m × 4.6 mm I.D. stainless-steel column containing Partisil silica gel 10 μm (Whatman, Clifton, N.J., U.S.A.).

Instrumental parameters. A DuPont Model 830 high-pressure liquid chromatograph equipped with a Model 835 multiwavelength UV detector operated at 254 nm and a Waters Assoc. loop injector Model No. U6K was used. A Schoeffel Model FS-970 fluorescence detector operated at 240 nm for excitation and at wavelengths greater than 350 nm for emission (Corning No. 0-52 filter) was used for fluorimetric detection. The isocratic mobile phase used was a mixture of methylene chloride–methanol–acetic acid (98:1:1) at a head pressure of 750 p.s.i. and a flow-rate of 1.5 ml/min. Under these conditions, the retention time of compound I was 3.8 min and that of compound II 5.2 min. The UV detector sensitivity was 1×10^{-2} a.u.f.s., and the fluorescence detector sensitivity was 0.1 μA.f.s. The chart speed on the 1.0-mV Honeywell recorder (Model No. 194) was 30 in./h. Under these conditions 200 ng of compound I and 150 ng of compound II per 10 μl injected give nearly full-scale pen response when operated in the UV mode, whereas 3 ng of compounds I and II per 10 μl injected give nearly full-scale pen response when operated in the fluorescence mode. The minimum detectable amounts of compounds I and II are 100 and 150 ng/ml of blood, respectively, using the UV detector and 3 ng/ml of blood using the fluorescence detector.

Analytical standards. Compound I ($C_{15}H_{12}ClNO_2$, MW = 273.72, m.p. = 192–194°) and compound II ($C_{15}H_{14}ClNO$, MW = 259.73, m.p. = 170–171.5°) of pharmaceutical grade purity (>99%) are used as analytical standards.

Prepare stock solutions of compounds I and II in separate 10-ml volumetric flasks by dissolving 10 mg of each compound into 1 ml of methanol. Dilute to volume with methylene chloride–acetic acid (99:1). These stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions (Table I) by suitable dilutions in methylene chloride–methanol–acetic acid (98:1:1), 100 μl of which are added to blood as internal standards.

Ten-microliter aliquots of solutions A to D or E to H are injected as external standards for establishing the HPLC parameters using either the UV or the fluorescence detector, respectively. Aliquots (100 μl) of the same solutions are added to blood as the internal standard calibration curve for the determination of the concentration in the unknowns and for the determination of percent recovery.

Calibration of compounds I and II by HPLC. A calibration (external standard) curve of the peak area ratio of compound I to compound II *vs.* the concentration of

TABLE I

STANDARD SOLUTIONS TO BE USED WITH AN UV AND A FLUORESCENCE DETECTOR

Standard	*Compound I*	*Compound II (ref. std.)*
UV detector (μg/100 μl)		
A	0.5	1.5
B	1.0	1.5
C	1.5	1.5
D	2.0	1.5
Fluorescence detector (ng/100 μl)		
E	7.5	30
F	15	30
G	22.5	30
H	30	30

compound I per 100 μl of methylene chloride–methanol–acetic acid (98:1:1) is constructed. A fresh calibration curve of the external standards and of the recovered internal standards are prepared for each day of analysis to establish the reproducibility of the HPLC system.

Reagents. All reagents must be of analytical reagent grade (>99% purity). Potassium phosphate buffer (1.0 *M*, pH 6) is prepared by mixing equal volumes of 1 *M* $K_2HPO_4 \cdot 3H_2O$ (228.23 g/l) and 1 *M* KH_2PO_4 (136.09 g/l). Mix well by inversion and check final pH with a pH meter. Absolute diethyl ether (Mallinckrodt, St. Louis, Mo., U.S.A.) is the extraction solvent, and a mixture of methylene chloride–methanol–acetic acid (98:1:1) is used for both the mobile phase for the HPLC system and to make standard solutions of compounds I and II.

Analysis of blood. The flow diagram of the extraction procedure is shown in Fig. 2.

Into a 15-ml conical centrifuge tube (PTFE No. 13 stoppered), add 0.5 ml oxalated whole blood, 2 ml of 1 *M* phosphate buffer (pH 6), mix well, and extract with 8 ml of diethyl ether by shaking for 10 min on a reciprocating shaker (Eberbach) at 80–100 strokes/min. Along with the samples, run a specimen of control blood and four 0.5-ml control blood specimens containing 0.1 ml of either standard solution A, B, C, or D (equivalent to 0.5, 1.0, 1.5, 2.0 μg of compound I and 1.5 μg of compound II per 0.5 ml blood) when using the UV detector or solutions E, F, G, or H (equivalent to 7.5, 15, 22.5, 30 ng of compound I and 30 ng of compound II per 0.5 ml blood) when using the fluorescence detector. Centrifuge the samples at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, rotor No. 253, Damon/IEC Corp.) at 5°. Repeat the extraction with another 8-ml portion of diethyl ether, centrifuge, and combine the ether extracts in a 15-ml conical centrifuge tube. Evaporate the ether extracts to dryness at 60° in a N-EVAP evaporator (Organomation Assoc.) under a stream of clean, dry nitrogen. Dissolve the residues in 100 μl of methylene chloride–methanol–acetic acid (98:1:1) and inject a 10-μl aliquot into the liquid chromatograph. Typical chromatograms of blood extracts are shown in Figs. 3 and 4.

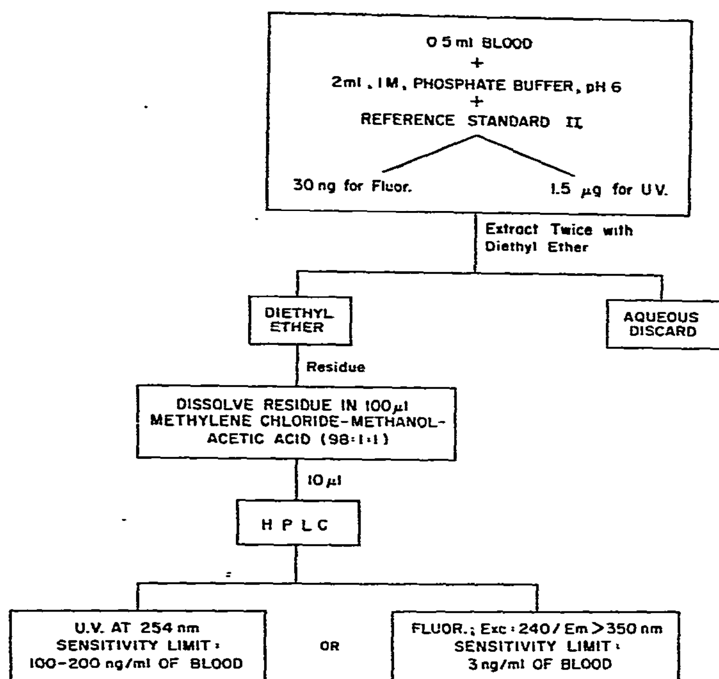

Fig. 2. Flow diagram of the extraction procedure for compounds I and II.

Calculations. The peak area ratio of compound I to compound II in the respective recovered internal standards is determined and plotted graphically *vs.* total concentration to establish the blood recovery curve. Similarly, the peak area ratio of compound I to compound II in the aliquots of the respective unknowns injected is also determined. The concentration of compound I in the unknowns represented by its peak area ratio is interpolated from the blood recovered internal standard curve. Since the peak area ratio of compound I to compound II is constant irrespective of the actual volume of sample injected or the total volume of the solvent, no dilution or aliquot factor is needed in the quantitation of the unknowns, even with further dilutions (*i.e.*, >100 μl), provided the peak due to the reference standard (compound II) is still measurable. The recovery factor for both internal and reference standards also remains constant throughout and is not needed for the calculation of the unknowns. Thus, concentration (ng) in the unknowns interpolated from the internal standard curve = ng of compound I per 0.5 ml of blood.

If, however, the peak due to the reference standard is diluted out, a direct calibration technique must be employed whereby a calibration curve of peak area of the recovered internal standard of compound I *vs.* concentration is plotted and used for the quantitation of the unknowns. Furthermore, the amount of compound I per aliquot of the unknown sample injected has to be corrected for the dilution of the total sample.

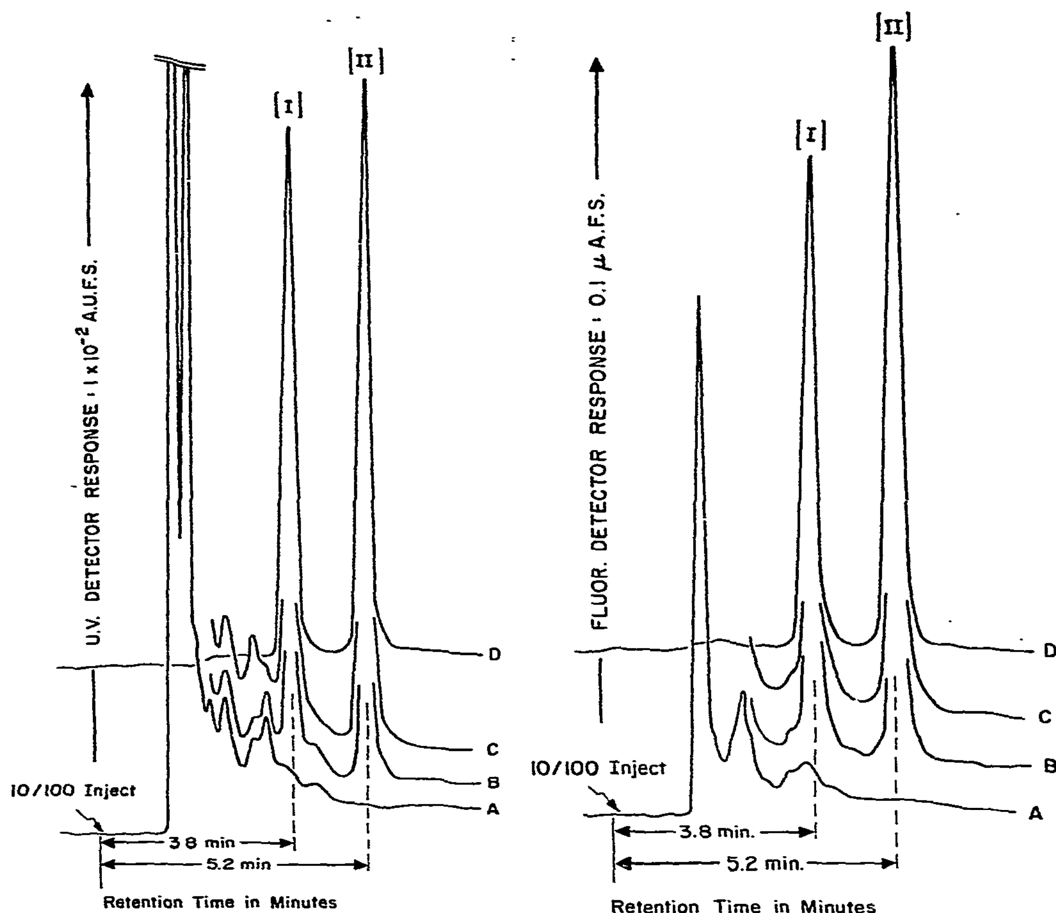

Fig. 3. Chromatograms of HPLC analysis, using a UV detector, of diethyl ether extracts of (A) control blood, (B) control blood containing added authentic standard, (C) subject blood post oral dose, and (D) authentic standard.

Fig. 4. Chromatograms of HPLC analysis, using a fluorescence detector, of diethyl ether extracts of (A) control blood, (B) control blood containing added authentic standard, (C) subject blood post oral dose, and (D) authentic standard.

RESULTS AND DISCUSSION

The intense UV absorption and luminescence properties of the carbazole class of compounds is well documented[5,6]. A sensitive and specific HPLC assay was developed for the determination of compound I from 1 ml or less of blood, employing either a UV or a fluorescence detector. This method provides for rapid and simple quantitation of compound I for routine analysis of the large number of samples obtained from clinical studies.

The major UV absorption bands of compounds I and II occur at 240–242 nm and are shown in Fig. 5. The DuPont Model 835 multi-wavelength UV detector was

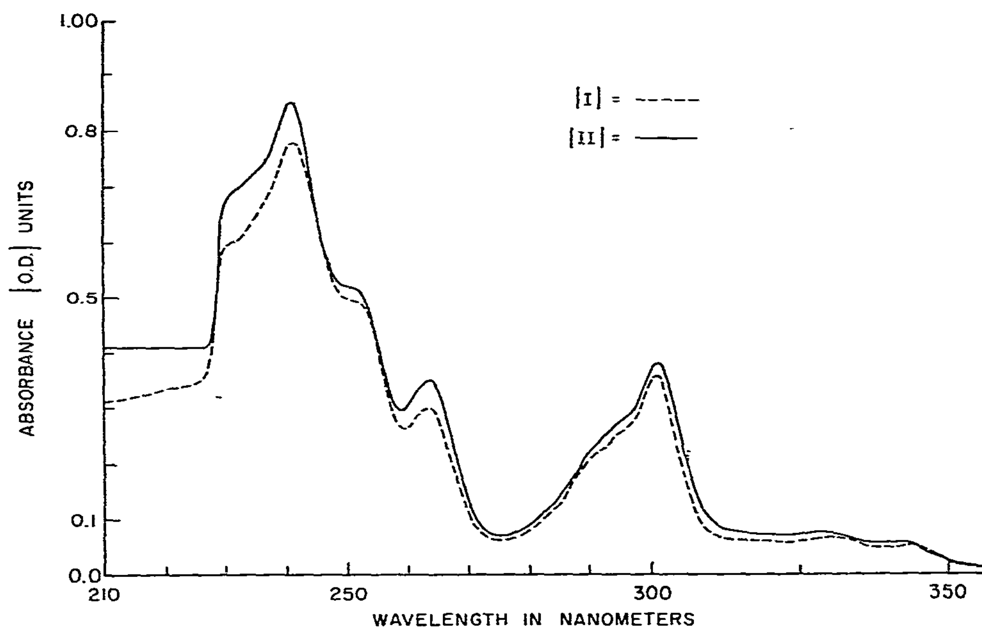

Fig. 5. UV absorption spectra of 4 μg/ml solutions of compounds I and II in methylene chloride–methanol–glacial acetic acid (98:1:1).

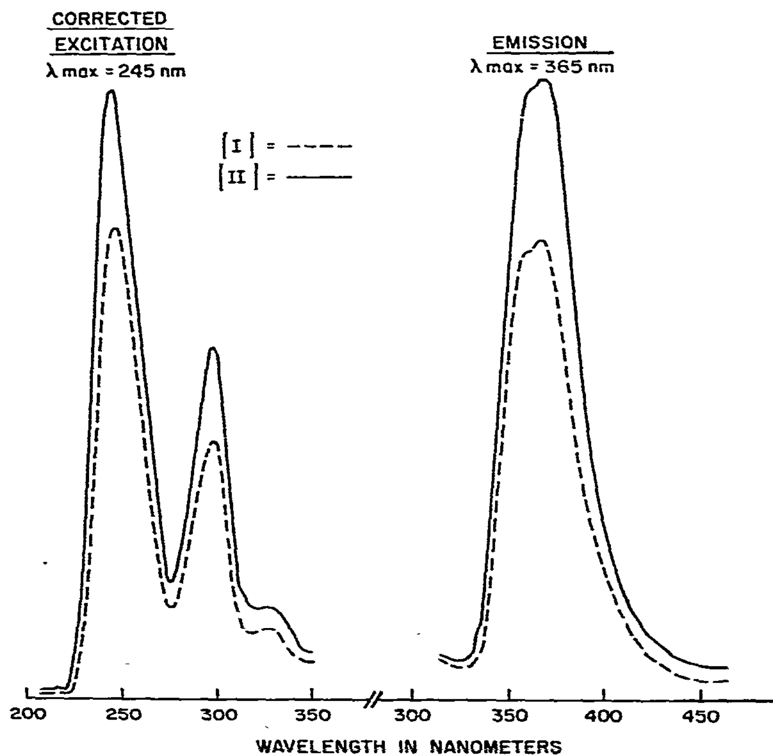

Fig. 6. Corrected excitation and emission spectra of 100 ng/ml solutions of compounds I and II in methylene chloride–methanol–glacial acetic acid (98:1:1).

used at 254 nm in conjunction with a low-pressure mercury lamp. Although measuring the UV absorption of compounds I and II at 254 nm is not at the maxima for both compounds (Fig. 5), the sensitivity at this wavelength was sufficient for the comparison of this method to the previously published luminescence methods[3,4]. If more sensitivity is required (approximately fifty fold), then the fluorescence detector should be used in tandem with the UV detector. The corrected excitation and emission spectra of compounds I and II using a Farrand spectrofluorometer equipped with a xenon lamp are shown in Fig. 6. The corrected excitation maximum is at 245 nm and coincides with the absorption maxima at 240–242 nm. The emission maximum is at 365 nm. The excitation monochromator of the Schoeffel Model FS970 fluorescence detector is set at 240 nm owing to the higher energy output of the deuterium lamp used as its energy source. A Corning No. 0-52 filter (greater than 350 nm band-pass) is used for measuring the fluorescence emission of both compounds.

The HPLC assay is the method of choice because it is a simple three-step operation that involves selective extraction, sample concentration, and direct analysis by HPLC. An earlier luminescence method[3] employs double extraction, a two step thin-layer chromatographic (TLC) separation and elution prior to fluorometric determination. The EC–GLC method[3], in addition to the above steps, also requires esterification prior to analysis.

TABLE II

COMPARISON OF THE HPLC METHOD USING A UV DETECTOR AND THE TLC–FLUORESCENCE METHOD

n.d. = Not detectable. Limit of sensitivity = 0.1–0.2 μg/ml blood. Correlation coefficient = 0.99.

Subject	*Day of dose*	*Time after a 100-mg dose t.i.d. (h)*	*Concentration of compound I in blood (μg/ml)*	
			HPLC	*TLC–fluorescence*
A	Day 14	0	1.63	1.93
		0.5	10.1	8.92
		1	6.42	6.09
		2	4.30	4.26
		5	2.00	2.10
		8	1.29	1.48
		12	0.86	1.13
		24	0.64	0.85
		48	0.32	0.48
		168	n.d.	n.d.
B	Day 1	0	n.d.	n.d.
		0.5	0.40	0.68
		1	2.99	3.81
		2	5.60	6.24
		5	2.70	3.04
	Day 3	0	3.01	3.63
		0.5	4.20	5.06
		1	7.19	7.67
		2	6.70	7.37
		5	4.83	5.11

Recovery and sensitivity limits of the HPLC assay

The overall recovery of compounds I and II from blood is of the order of 97.3 ± 5.3% (S.D.). The sensitivity limit of detection is 100–200 ng/ml of blood, using a UV detector at 254 nm, or 3 ng/ml of blood using a fluorescence detector with excitation at 240 nm and emission at wavelengths greater than 350 nm.

Application of the method to biological specimens

In order to evaluate the clinical utility of the HPLC method, it was necessary to analyze blood samples obtained from clinical studies on compound I that were previously analyzed by the TLC–fluorescence method[3].

The HPLC method was used to monitor the blood level–time profiles in two subjects following 14 consecutive days of oral dosing at 100 mg three times a day. One subject was monitored for 168 h, following the last dose on day 14. A second subject was monitored only on days 1 and 3 following the first daily dose of compound I The blood levels obtained by the HPLC method were statistically compared with those obtained by the TLC–fluorescence method using linear regression analysis. The

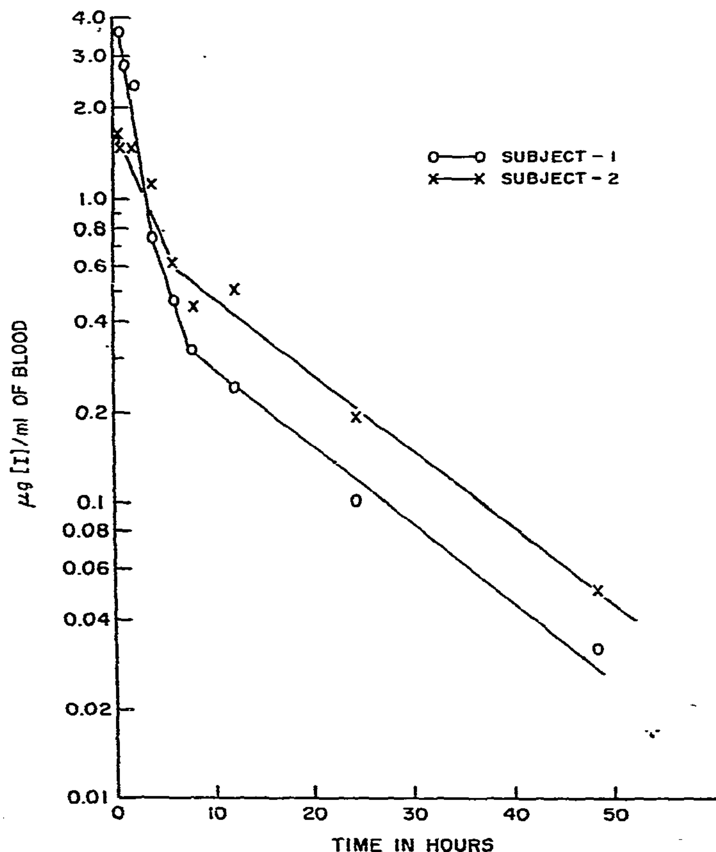

Fig. 7. Blood level fall-off curves in man following the oral administration of a single 100-mg dose of compound I.

resulting least squares line ($r = 0.99$) indicated that a slope of 1 and an intercept of 0 fell within the 95% confidence limits. The results and comparison of the two methods are shown in Table II. The HPLC method was also applied to the analysis of blood samples following a single 100-mg oral dose of compound I in two subjects (Fig. 7). The blood level–time curves from these two subjects show peak levels at 0.5 h post administration of 3.6 and 1.6 μg/ml of blood, respectively, indicating rapid absorption of the drug. The blood levels at 24 and 48 h in both subjects were below the limit of quantitation of the UV detector, hence required the sensitivity of the fluorescence detector for their quantitation.

ACKNOWLEDGEMENTS

We wish to thank Mr. R. McGlynn for the drawings of the figures presented and Mrs. A. Szilagyi for typing this manuscript.

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