Journal of Chromatography, 274 (1983) 139—148
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1626

GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF TOLOXATONE IN HUMAN PLASMA

ROUTINE ANALYSIS OF A WIDE RANGE OF DRUG CONCENTRATIONS USING A NITROGEN-SELECTIVE DETECTOR

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(First received October 20th, 1982; revised manuscript received December 30th, 1982)

SUMMARY

A selective and sensitive gas—liquid chromatographic (GLC) method has been developed for the measurement of toloxatone at therapeutic concentrations in plasma. The technique is based on a single extraction from plasma at pH 10, the preparation of a trimethylsilyl derivative and detection by a nitrogen-selective detector. The traditional calibration curve, peak-area ratio of toloxatone to internal standard versus toloxatone plasma concentration, was slightly concave for the wide concentration considered (10—3000 ng/ml). As a consequence, the linear least-squares regression analysis gave a negative intercept on the *y*-axis which affected quantitation accuracy of low plasma concentration values. A calibration method taking into consideration the non-linearity of the calibration curve is proposed.

The characteristics of the detector were examined in order to analyse response linearity and sensitivity. A linear relationship was found between background current and detector sensitivity.

INTRODUCTION

Toloxatone, 5-(hydroxymethyl)-3-(3-methylphenyl)-2-oxazolidinone is a reversible inhibitor of type A monoamine oxidase both in vitro and ex vivo in the rat [1] and has been shown to possess an antidepressant activity in man [2—4]. Two thin-layer chromatographic (TLC) methods for the determination of toloxatone in plasma have been reported [5, 6]. In the first technique [5] [^{14}C]toloxatone was measured by liquid scintillation counting after TLC separation of the drug from metabolites. The technique was therefore unsuitable for studies with unlabelled drug. The second analytical method [6] was based on TLC of a dichloroethane—amyl alcohol plasma extract followed

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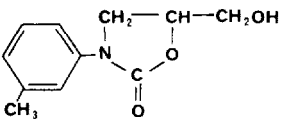
by densitometric quantitation. The detection limit of this method was 10 ng/ml with a coefficient of variation of 12% at a concentration of 250 ng/ml. This technique was time-consuming and required a large volume of plasma (5 ml).

The aim of the present investigation was to improve the precision and the sensitivity of the quantitative analysis of toloxatone in plasma using a simple gas—liquid chromatographic (GLC) method.

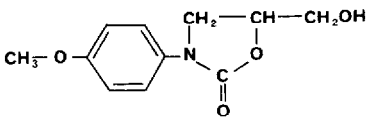
EXPERIMENTAL

Standards and reagents

Toloxatone and the internal standard MD700075, 5-(hydroxymethyl)-3-(4-methoxyphenyl)-2-oxazolidinone, were synthesized in the Chemistry Department of Delalande Research Centre. The chemical structures are given in Fig. 1. All reagents were of analytical grade purity. Toluene and ethyl acetate were RPE-ACS grade, and diethyl ether was RPE grade (Carlo Erba, Milan, Italy). Ethyl acetate was dried over anhydrous Na_2SO_4 (Merck, Darmstadt, G.F.R.); sodium hydroxide (Prolabo, Paris, France) and phenazine (Merck) were used. Trimethylsilyl derivatives were prepared with N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Pierce, Rockford, IL, U.S.A.).



TOLOXATONE



MD 700075

Fig. 1. Structures of toloxatone and internal standard.

Gas—liquid chromatographic conditions

A 5880A Hewlett-Packard gas chromatograph (with a Model Level 4 Terminal) equipped with a nitrogen—phosphorus detector (NPD) was used. A glass column (2 m × 2 mm I.D.) was silanized and packed with 3% OV-101 coated on Supelcoport 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.). A second column of the same geometry was packed with 3% SP-2250 coated on Supelcoport 80—100 mesh (Supelco). Both columns were conditioned as follows: a temperature program was run from 50°C to 300°C at 5°C/min flushing the column with nitrogen at a flow-rate of 40 ml/min, then the column was held isothermally at 300°C for 2 h without gas flow and for 48 h with a nitrogen flow-rate of 40 ml/min.

The operating conditons were: column temperature 200°C (OV-101) or 235°C (SP-2250), with injection port and detector temperature at 300°C. Helium was used as carrier gas at a flow-rate of 35 ml/min.

Gas chromatography—mass spectrometry

Identification of the gas chromatographic peaks was made by gas chromatography—mass spectrometry (GC—MS) under the electron-impact mode with a Nermag R10-10 mass spectrometer coupled with a Sidar data system and with a Girdel gas chromatograph (Model 32). Operating conditions were: electron beam energy 70 eV, filament current 200 μA, pressure of the ion source $2 \cdot 10^{-2}$ Torr and ion-source temperature of 170°C. Data were acquired for ions in the mass range of 50—550 a.m.u. A glass column (2 m × 2 mm I.D.) packed with 3% SP-2250 coated on Supelcoport 100—120 mesh (Supelco) was coupled to the mass spectrometer. The temperature of the interface was 260°C.

Sample preparation

A standard solution of internal standard MD700075 (10 ng/μl) was prepared in distilled water. For the calibration curves, aqueous solutions of toloxatone (10, 50, 100, 500, 1000, 2000 and 3000 ng per 200 μl) were prepared using the aqueous solution of the internal standard MD700075 (10 ng/μl).

The extraction recovery of toloxatone was determined by addition of the internal standard after the extraction of the drug from plasma (reference sample was the same sample without extraction).

Among the solvents tested (toluene, ethyl acetate and diethyl ether) toluene at pH 10 provided the cleanest plasma extract with a recovery of 73 ± 2% (n = 3).

To a conical tapered tube, 200 μl of internal standard solution (for the calibration curve 200 μl of internal standard—toloxatone solution), 1 ml of plasma and 110 μl of 0.2 N sodium hydroxide solution (to adjust pH to 10) were added and vigorously shaken on a Vortex mixer. The sample was extracted with 6 ml of toluene for 30 min on a rock-and-roll extractor. The two phases were then separated by centrifugation at 4°C (1000 g for 10 min). The aqueous phase was discarded and the toluene phase was transferred to another tube and evaporated to dryness at 55°C in a water bath. Then 100 μl of a solution of BSTFA—ethyl acetate (1:5) were added. After 30 sec shaking on a Vortex mixer and 30 min reaction time at 60°C the excess reagent was evaporated to dryness at 40°C under a stream of nitrogen. The dry extract was then dissolved in 100 μl of ethyl acetate (shaking on a Vortex mixer for 1 min); 2 μl of this sample were injected into the gas chromatograph. No significant degradation of the trimethylsilyl derivatives of toloxatone and its internal standard was noticed after ten days at 4°C.

Statistical analysis

Calibration curves were tested using a programme for regression analysis [7] with a Tektronix 4052 desk computer. In the routine assay a Texas TI-51-III calculator was used.

RESULTS AND DISCUSSION

Gas chromatographic separation

Typical chromatograms of plasma extracts of toloxatone and its internal standard are shown in Figs. 2 (OV-101) and 3 (SP-2250).

Peak symmetry and resolution for both compounds were improved by formation of trimethylsilyl (TMS) derivatives. The retention time of the TMS derivatives of toloxatone (Tx-TMS) and the internal standard (IS-TMS) were adjusted approximately to 4 and 8 min on the SP-2250 column and to 2.5 and 4.5 min on the OV-101 column, respectively. Under these conditions the peaks corresponding to Tx-TMS and IS-TMS were well resolved. Overall time of analysis was 10 min on the SP-2250 column and 6 min on the OV-101 column.

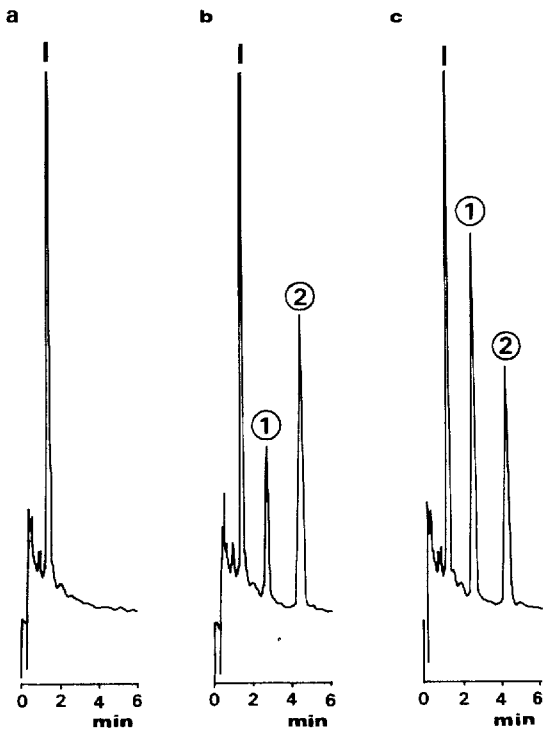


Fig. 2. Chromatograms obtained with the 3% OV-101 column at 200°C. (a) Plasma extract of a blank sample; (b) plasma extract of a sample containing spiked concentrations of toloxatone (500 ng/ml) and MD700075 (2000 ng/ml); (c) typical chromatogram after oral administration of toloxatone. 1 = Tx-TMS; 2 = IS-TMS; I = caffeine.

Derivatisation was checked by GC—MS. The mass spectra of the gas chromatographic peaks corresponding to Tx-TMS (molecular ion *m/z* 279) and IS-TMS (molecular ion *m/z* 295) demonstrated that both compounds were monosilylated. As shown in Fig. 2 and 3, an unknown compound was extracted from plasma samples (compound I). This peak was identified as caffeine using the library search programme of the Sidar data system.

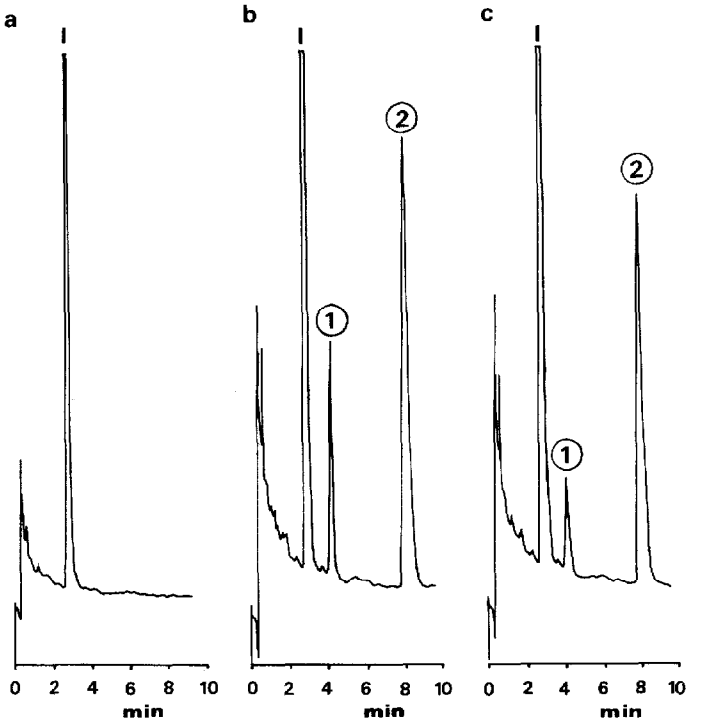


Fig. 3. Chromatograms obtained with the 3% SP-2250 column at 235°C. (a) Plasma extract of a blank sample; (b) plasma extract of a sample containing spiked concentrations of toloxatone (500 ng/ml) and MD700075 (2000 ng/ml); (c) typical chromatogram after oral administration of toloxatone. 1 = Tx-TMS; 2 = IS-TMS; I = caffeine.

Evaluation of the NPD

Since the first description by Kolb and Bischoff in 1974 [8], the NPD has been extensively used for quantitative analysis of drugs in body fluids and in tissues by GLC. However, in spite of its widespread use, its mechanism has not been yet fully elucidated [9, 10]. The NPD is known to have a relatively high baseline and sensitivity drift. In addition, there is a loss of sensitivity of the rubidium ceramics with time and so it is not easy to maintain the same sensitivity [11]. These problems can be minimised using an internal standard assuming the same change of sensitivity for the drug measured and for the reference compound. This problem was investigated by measuring the detector response of toloxatone/internal standard TMS derivatives at different heating power values.

Detector background current but not heating power was linearly related to detector response (Fig. 4). A practical consequence of this linear relationship of background current against detector response is that, with an internal standard, the heating power of the NPD may be increased occasionally for analysis of low concentrations. Relative detector response for toloxatone and the internal standard was determined from the slope of the two curves and gave a value of 1.03, the theoretical value calculated from their molecular weights being 1.08.

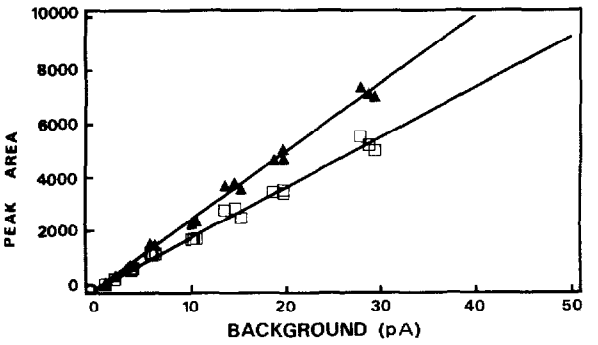


Fig. 4. Relationship between detector background current (pA) and detector response (peak area). Injection of Tx-TMS (▲, 40 ng toloxatone) and IS-TMS (□, 30 ng internal standard). Intercept values are not significantly different from the detector zero current. ▲: $y = 258.1x - 197.3$, $r = 0.9971$, $x_{(y=0)} = 0.76$ pA. □: $y = 188.0x - 123.1$, $r = 0.9948$, $x_{(y=0)} = 0.65$ pA.

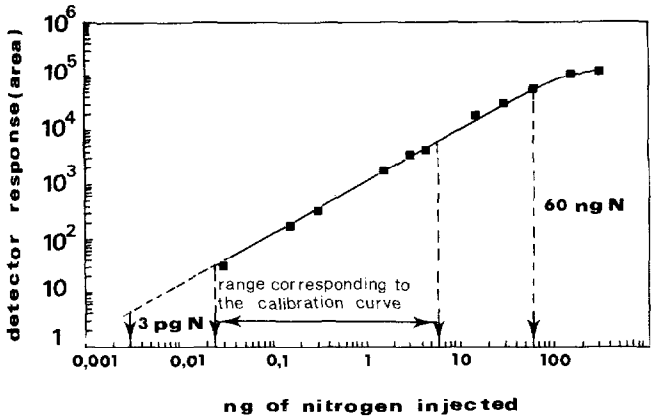


Fig. 5. NPD response linearity with phenazine (for conditions, see text).

NPD sensitivity was tuned by repeated injections of phenazine (SP-2250 column at 190°C). Optimum sensitivity and the same time-stable function of the detector was obtained with a hydrogen flow rate of approx. 2 ml/min and an air flow rate of approx. 45 ml/min. The lower detection limit using phenazine was estimated to be 3 pg of injected nitrogen. The linear dynamic range was approx. 10^4 (Fig. 5).

Calibration curves

Quantitative analysis was carried out using the area ratio method. Area ratios (y = Tx-TMS/IS-TMS) were plotted against concentration of toloxatone (x). Slope and intercept of the calibration curves were determined by linear regression analysis. The precision (relative standard deviation) and the accuracy (relative error) of the method were established in the range of toloxatone concentrations from 10 to 3000 ng/ml. Each sample was analysed in triplicate. Tables I and II show the results obtained with the same samples using SP-2250 and OV-101.

The negative y-intercept using the OV-101 column made this calibration

TABLE I

PRECISION AND ACCURACY OF THE GLC METHOD FOR THE MEASUREMENT OF TOLOXATONE

Column: 3% OV-101, 200°C. $n = 3$.

	Spiked concn. (ng/ml) (x)	Relative S.D. (%) (y)	Found concn. (ng/ml) (x')	S.D. (ng/ml) (x')	Relative S.D. (%) (x')	Mean error $\Delta x = x' - x$ (ng)	Relative error $\Delta x/x$ (%)
Straight-line fit	10	4.2	73.2	0.5	0.7	+ 63.2	+ 632.0
$y = 0.001067x - 0.06913$	50	4.4	102.0	1.8	1.8	+ 52.0	+ 104.0
($r = 0.9967$)	100	4.4	138.6	5.0	3.6	+ 38.6	+ 38.6
	500	3.7	449.0	14.5	3.2	− 5.1	− 10.2
	1000	0.8	876.0	5.9	0.7	− 124.0	− 12.4
	2000	4.5	1924.0	84.0	4.4	− 76.0	− 3.8
	3000	2.7	3098.0	84.0	2.7	+ 98.0	+ 3.2
Power-function fit	10	4.2	11.3	0.6	5.5	+ 1.3	+ 13.0
$y = 0.00073x^{1.0330}$	50	4.4	47.5	2.2	4.6	− 2.5	− 5.0
($\log y = 1.0330 \log x - 3.1342$)	100	4.4	91.9	4.2	4.5	− 8.1	− 8.1
($r = 0.9988$)	500	3.7	456.2	16.4	3.6	− 43.8	− 8.7
	1000	0.8	940.4	7.1	0.7	− 59.6	− 5.9
	2000	4.5	2099.5	92.0	4.4	+ 99.5	+ 5.0
	3000	2.7	3372.0	89.6	2.6	+ 372.0	+ 12.4

TABLE II

PRECISION AND ACCURACY OF THE GLC METHOD FOR THE MEASUREMENT OF TOLOXATONE

Column: 3% SP-2250, 235°C. $n = 3$.

	Spiked concn. (ng/ml) (x)	Relative S.D. (%) (y)	Found concn. (ng/ml) (x')	S.D. (ng/ml) (x')	Relative S.D. (%) (x')	Mean error $\Delta x = x' - x$ (ng)	Relative error $\Delta x/x$ (%)
Straight-line fit	10	6.0	36.2	0.5	1.4	+ 26.2	+ 262.0
$y = 0.00090x - 0.02491$	50	3.1	70.6	1.3	1.9	+ 20.6	+ 41.2
($r = 0.9991$)	100	5.0	113.4	4.3	3.8	+ 13.4	+ 13.4
	500	1.9	479.3	8.5	1.8	− 20.7	− 4.1
	1000	2.1	950.8	19.7	2.0	− 49.2	− 4.9
	2000	4.9	1971.6	96.0	4.8	− 28.4	− 1.4
	3000	1.6	3037.6	48.4	1.6	+ 37.6	+ 1.2
Power-function fit	10	6.0	10.3	0.6	5.8	+ 0.3	+ 3.0
$y = 0.00070x^{1.0283}$	50	3.1	49.7	1.5	3.0	− 0.3	− 0.6
($\log y = 1.0283 \log x - 3.1572$)	100	5.0	97.3	4.7	4.8	− 2.7	− 2.7
($r = 0.9998$)	500	1.9	489.7	9.0	1.8	− 10.3	− 2.0
	1000	2.1	981.1	20.3	2.0	− 18.9	− 1.9
	2000	4.9	2023.7	96.8	4.8	+ 23.7	+ 1.2
	3000	1.6	3096.3	48.9	1.6	+ 96.3	+ 3.2

curve unsuitable for the low toloxatone concentrations, as this linear regression would severely overestimate the lowest concentration values (Table I). The situation was improved when using the SP-2250 column; however, even this linear regression gave unacceptable errors for low concentrations (Table II). Note that the correlation coefficients calculated from all experimental data points were 0.9991 and 0.9967 for SP-2250 and OV-101, respectively. These values emphasize the caution needed when this parameter is used to judge linearity.

TABLE III

CHARACTERISTICS OF THE CALIBRATION CURVE BY INCREASING THE RANGE OF TOLOXATONE PLASMA CONCENTRATIONS

Column: 3% SP-2250, 235° C.

Range of calibration (ng/ml)	Sraight-line fit ($y = ax + b$)				Power-function fit $y = ax^b$ (log y = log a + $b$log x)*			
	$ax \cdot (10^{-4})$	$bx \cdot (10^{-4})$	$x_{(y=0)}$ (ng)	r	log a	$a \cdot 10^{-4}$	b	r*
10— 50	7.74	0.72	0.09	0.9988	—3.1207	7.57	1.0052	0.9988
10— 100	7.72	0.07	0.01	0.9977	—3.1180	7.62	1.0029	0.9992
10— 500	8.16	22.3	2.7	0.9997	—3.1340	7.35	1.0143	0.9997
10—1000	8.31	40.5	4.9	0.9997	—3.1397	7.25	1.0180	0.9998
10—2000	8.72	141.9	16.0	0.9985	—3.1493	7.09	1.0238	0.9998
10—3000	9.00	249.1	27.7	0.9991	—3.1572	6.96	1.0283	0.9998

*Linear regression after logarithmic transformation.

Table III shows that slope and intercept on the y-axis increased by increasing the range of concentration values, indicating that calibration curves were slightly concave.

The possible cause of this non-linearity could be: loss by adsorption in the glassware, adsorption in the chromatographic system and/or in the extraction procedure, non-linear detector signal and integration errors. Some of these factors can be controlled; however, it is not always possible to determine the step which causes the non-linearity.

The comparison between the results obtained from the two different columns suggests an important contribution of the GLC system to the non-linearity of the calibration curve.

In spite of a better peak symmetry when using OV-101 column, the SP-2250 column gave a better linearity, probably because of a better deactivated column packing.

Non-linear calibration curves can be used by dividing them into linear sub-ranges [12] or by polynomial fitting [13]. With our data a power function through the origin ($y = ax^b$) linearized by a logarithmic transformation (log y = log a + $b$log x) was attempted. The advantage of this fitting is that the

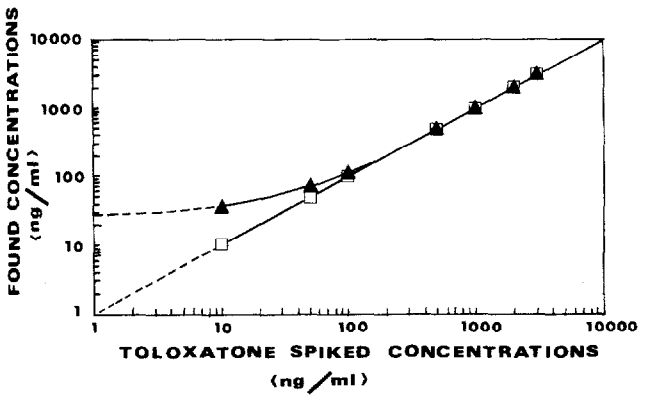


Fig. 6. Accuracy of the calibration (SP-2250 column). (▲), linear calibration curve; (□), power-function fit calibration curve with logarithmic transformation.

logarithmic transformation introduces a weighting factor in the least-squares computations, which is inversely proportional to the observed value. This weighting factor seems to be reasonable, because it corresponds to the hypothesis of approximately constant relative standard deviation of the observed area ratio values (S.D.$(y)/\bar{y}$).

The comparison between results from the two methods of calculation (Tables I and II) showed that the power function fitting linearized by logarithmic transformation considerably improved the accuracy of the low concentrations (Fig. 6).

The day-to-day reproducibility of the calibration curves was investigated during one month and the overall relative standard deviation was $\leqslant$ 6% for toloxatone plasma concentrations between 10 and 3000 ng/ml.

Application of the method

This method has been extensively applied in our laboratory to investigating the pharmacokinetics of toloxatone in man following single and multiple doses. A typical drug plasma concentration—time plot after oral administration of a 200-mg capsule to a healthy adult is shown in Fig. 7.

Toloxatone plasma concentrations were determined in the 0—12 h interval using both the linear and the logarithmic calibration curves. No difference of toloxatone plasma concentrations was observed above 100 ng/ml. However, when plasma concentrations were below 100 ng/ml the linear calibration curve gave a significant overestimation of the concentration values and this would considerably change the interpretation of the experimental data.

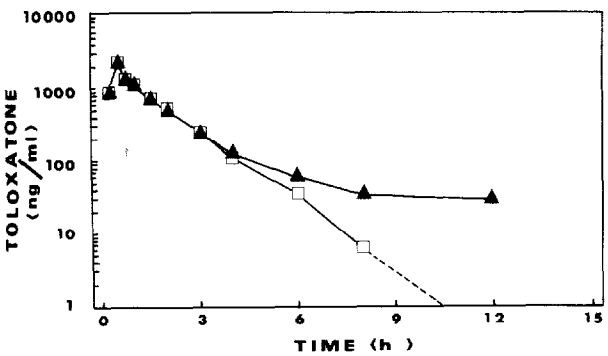


Fig. 7. Plasma toloxatone concentrations from a healthy adult following a single oral administration of the drug (200 mg, capsule). (▲), linear calibration curve; (□), power-function fit calibration curve with logarithmic transformation.

ACKNOWLEDGEMENTS

We wish to thank Dr. C. Gomeni for her advice in mathematics and Mr. M. Sanjuan for mass spectral data. Mrs. M. Farny provided excellent assistance in the preparation of this manuscript.

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