Journal of Chromatography, 181 (1980) 41—49
Biomedical Applications

CHROMBIO. 423

PAIRED-ION CHROMATOGRAPHIC ANALYSIS OF TAMOXIFEN AND TWO MAJOR METABOLITES IN PLASMA

YECHIEL GOLANDER and LARRY A. STERNSON*

Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kans. 66045 (U.S.A.)

(First received May 8th, 1979; revised manuscript received August 21st, 1979)

SUMMARY

A method is described for the clinical analysis of the non-steroidal anti-estrogenic, anti-neoplastic agent, tamoxifen and its 4-hydroxy and N-desmethyl metabolites in human plasma. The analytes are extracted from biological fluid with diethyl ether and subsequently converted to fluorescent phenanthrene derivatives by irradiation with UV light. The fluorophores are separated by paired-ion chromatography on a reversed-phase (C_{18}) column. Spectrofluorometric monitoring of the column eluent allows quantitation of analytes as their phenanthrene derivatives to levels of 100 pg/ml of plasma.

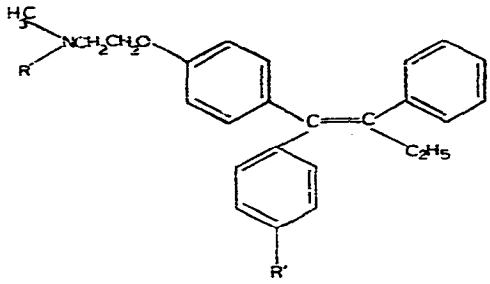
INTRODUCTION

Tamoxifen (I; ICI 46,474 or Nolvadex, Imperial Chemical Industries, Macclesfield, Great Britain) is a non-steroidal anti-estrogenic compound being used successfully in the treatment of metastatic breast cancer [1—5]. We recently described [6] an analytical method for monitoring tamoxifen and its 4-hydroxy derivative (II) in plasma. After extraction from biological fluid, the analytes are photochemically converted to fluorescent phenanthrene derivatives which are then separated by liquid chromatography on a μBondapak CN column. Spectrofluorometric monitoring of the column eluent allows the detection of approximately 2 ng of analyte per ml of biological fluid.

This method has now required modification in light of recent findings by Adam et al. [7] indicating that a major route of detoxication of tamoxifen (in addition to aromatic hydroxylation [8]) involves hepatic N-demethylation producing desmethyltamoxifen (III) and that this metabolite may in itself possess

*To whom correspondence should be addressed.

anti-estrogenic activity. Furthermore, the fluorescent products formed from I and II were somewhat unstable in the photolysis media, requiring that reaction times be strictly controlled (± 2 min) to avoid loss in fluorescence during reaction.



I : R = CH_3, R' = H
II : R = CH_3, R' = HO
III : R = H, R' = H

MATERIALS AND METHODS

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A solvent delivery system, Model U6K injector, Model 440 dual-channel absorbance detector, operated at 254 nm, and a Schoeffel FS 970 fluorescence LC detector (Schoeffel, Westwood, N.J., U.S.A.). The excitation monochromator of this detector was fixed at 256 nm and emitted radiation was passed through a filter with 340-nm cut-off. A 30 cm × 4.6 mm I.D. μBondapak C_{18} 10-μm particle size column (Waters Assoc.) was used for all separations.

Materials

Tamoxifen, 4-hydroxytamoxifen (II) and desmethyltamoxifen (III) were provided by Imperial Chemical Industries and used without further purification. Mobile phase for high-performance liquid chromatography (HPLC) was prepared with Fisher HPLC grade methanol (Fairlawn, N.J., U.S.A.). Sodium pentanesulfonate was purchased from Eastman (Rochester, N.Y., U.S.A.) and used as obtained.

Procedure

Samples (5 ml) of fresh plasma containing known amounts of I, II and III (prepared by the addition of 25—100-μl aliquots of methanolic stock solutions of I, II or III to 5 ml of plasma) were transferred to 45-ml, heavy-duty screw cap (with PTFE liner) centrifuge tubes and extracted once with 25 ml of diethyl ether (AR) for 20 min. The phases were subsequently separated by centrifugation of the mixture (1300 *g* for 15 min) and the aqueous layer was frozen in a dry-ice—acetone bath. An aliquot of the diethyl ether layer (20 ml) was then removed and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with vortex mixing in 1 ml of HPLC mobile phase [methanol—water (73:27) containing 0.5% acetic acid and 2.5 m*M* sodium pentanesulfonate].

Photochemical activation

This solution was then transferred to 1-cm^2 quartz cuvettes and irradiated for 20 min with a 15-W Hg vapor lamp (maximum output at 253.7 nm; General Electric No. G15T8) placed 10 cm from the quartz reaction vessel. Optimum reaction conditions were determined by irradiating solutions of tamoxifen, II and III, in various solvent mixtures, varying the distance between the lamp and the cell, and monitoring fluorescent intensity as a function of time.

Chromatographic analysis

A 50—200-μl aliquot of the reaction mixture was then chromatographed on a μBondapak C_{18} column. Components were eluted isocratically with a mobile phase consisting of methanol—water (73:27) containing 0.5% acetic acid and 2.5 m*M* sodium pentanesulfonate.

Components were quantitated as the photolyzed product by measuring peak area and comparing it with a standard curve constructed after analysis of plasma samples containing known amounts of I, II or III over the concentration range of 0.1—500 ng/ml of plasma (i.e. 0.1, 0.5, 1.0, 5.0, 10, 50, 100, 250, 500 ng/ml). Calibration curves used to validate the assay were thus prepared from samples containing nine different concentrations of analyte (and a blank). Four samples were prepared at each concentration and each sample was analyzed in triplicate. For routine assays only five concentrations were used (0.1, 1.0, 5.0, 50, 250 ng/ml) to prepare calibration curves and samples were prepared and analyzed in duplicate.

RESULTS

Extraction procedures

Tamoxifen (I) and its 4-hydroxy (II) and desmethyl (III) metabolites were removed from plasma by a single extraction with 4 volumes of diethyl ether. This extraction afforded an average recovery of 89 ± 4% for tamoxifen, 97 ± 4% for II and 83 ± 4% for III over the concentration range stated. Recoveries at specific concentrations are provided in Table I. During extraction, I and III are stable; however, II is converted to the corresponding phenanthrene derivative to an extent of ca. 50% over the 20-min extraction period. This decomposition does not affect the overall analysis, since II is ultimately quantitated as the corresponding phenanthrene. Chloroform, methylene chloride and ethyl acetate were poorer extractants for these analytes, having less favorable partition characteristics and being less selective (i.e. resulting in co-extraction of additional contaminants). Less polar solvents proved unsuitable since they resulted in reduction in extraction efficiency of I, II and III, apparently due to adsorption of analytes to container surfaces in these more lipophilic solvents, as has been reported by Thakker et al. [9] for other hydrophobic drugs. Ion-pair extraction [6] of I, II and III (in plasma adjusted to pH 1 with sulfuric acid) with diethyl ether and 0.1 *M* trichloroacetate offered no advantage over direct extraction and in fact was more time consuming and produced a more cluttered chromatogram. Addition of ion-pairing agents did improve the extractability of I, II and III into non-polar solvents (as previously seen by Thakker et al. [9], but not to efficiencies obtained with diethyl ether as extractant.

TABLE I

RECOVERY OF TAMOXIFEN AND METABOLITES FROM PLASMA

Concentration* (ng/ml) in plasma	Recovery (%)**,***		
	Compound I	Compound II	Compound III
0.10§	86 ± 6	91 ± 5	87 ± 5
0.50	91 ± 5	96 ± 3	88 ± 4
1.0	87 ± 1	101 ± 5	86 ± 2
5.0	96 ± 2	94 ± 2	79 ± 5
10.0	86 ± 5	95 ± 4	83 ± 2
50	92 ± 3	98 ± 3	85 ± 1
100	84 ± 4	100 ± 2	78 ± 5
250	90 ± 3	96 ± 2	79 ± 2
500	88 ± 1	104 ± 5	80 ± 3

* Known amount of analyte added to known volume of human plasma, as described in Materials and methods.

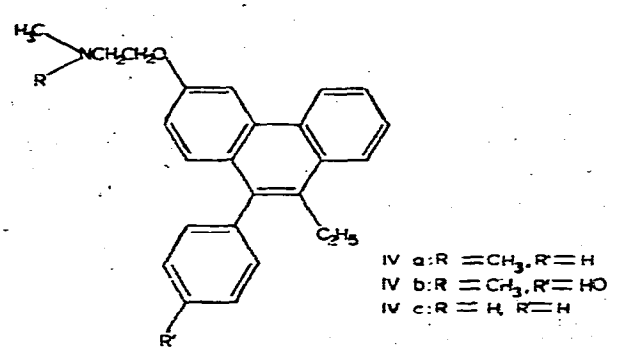
** Determined as the ratio of the area of the analyte peak obtained after carrying out the assay on a plasma sample (5 ml) spiked with a known amount of I, II or III; to the area of the analyte peak resulting after carrying out the assay on the same amount of I, II or III added to the mobile phase (5 ml).

*** Represents the average recovery ± S.D. for four plasma samples containing the same known concentration of analyte. Each sample is analyzed chromatographically in triplicate.

§ Represents the detection limit, defined as the minimum detectable fluorescent intensity which is three times the baseline level.

Photochemical conversion of analytes to fluorescent products

Tamoxifen, II and III exhibit no native fluorescence. We have previously demonstrated [6] that irradiation of solutions of I or II with UV light converts these materials to the corresponding phenanthrenes (IVa and b) which are strongly fluorescent (λ_{ex} = 256 nm; λ_{em} = 320 nm). These products (IVa and b) degrade in the photolysis media resulting in a loss of fluorescence with time [6]. Photochemical activation of III appeared to proceed through the identical reaction sequence, producing the corresponding fluorescent phenanthrene (IVc), which then decomposed. The addition of a small amount of acid to the photolysis media significantly inhibited the degradation of IVa—c to non-fluorescent products. The stabilizing influence of acid was observed in various photolysis media [dioxane—heptane (70:30); methanol; HPLC mobile phase]



IV a: R = CH_3, R' = H
IV b: R = CH_3, R' = HO
IV c: R = H, R' = H

and held for IVa—c. HPLC mobile phase was selected as the reaction medium since it offered maximum yield and stability of IVa—c and subsequently gave rise to the best chromatographic properties. Irradiation time and distance of the reaction vessel from the lamp were adjusted to maximize the rate of formation of fluorophores while minimizing degradation of the fluorophores in a manner analogous to that previously described [6]. An irradiation distance of 10 cm was chosen and photolysis carried out for 20 min. Conversion to phenanthrene was quantitative under these conditions. The formation of IV followed first-order kinetics (Fig. 1). The rate constants for formation of IV from I, II, and III are given in Table II.

TABLE II

RATE OF FORMATION OF PHENANTHRENES (IV) FROM PHENYLSTILBENES

Irradiation carried out in methanol—water (73:27) containing 0.5% acetic acid and 2.5 m*M* pentanesulfonic acid. Distance between lamp and sample cuvette was 10 cm. Reaction carried out as described in experimental sections.

Reaction*	k** (min^{-1})	$t_{½}$ (min)
I → IVa	0.15	4.6
II → IVb	0.29	2.4
III → IVc	0.11	6.5

* I, II, III present initially at concentration of 50 ng/ml.
** Reaction followed first-order kinetics.

Reaction was thus complete in 30 min. Degradation of IV ($\leq$ 10%) was observed only after photolysis had proceeded for 40 min; therefore, photolysis conditions need not be adhered to as strictly as those previously described [6]. Fig. 1 also shows that the fluorescence intensity of IVa is approximately 1.2 times that of IVc and 0.8 times that of IVb. The reason for this difference in relative intensities has not been determined but apparently resides with differences in either the molar absorptivity of the stilbenes or phenanthrenes or quantum yield of the latter.

Chromatographic analysis

Separation of components (I, II, III, IVa—c) could not be accomplished by reversed-phase or normal-phase chromatography, because of the inability to resolve compound IVb and IVc. However, using paired-ion chromatography with a reversed-phase (C_{18}) column, separation of tamoxifen species and corresponding phenanthrenes in biological samples could be accomplished. Using methanol—water mixtures and alkylsulfonate ion-pairing agents, the capacity factors (k') for IVa—c were most sensitive to change over the range 70—80% methanol, varying from ca. $k' = 1.9$ to 5 for tamoxifen and over a similar range for IVb and IVc. The system was much less sensitive to the nature of the ion-pairing agent. Using a mobile phase of methanol—water (80:20) k' for tamoxifen was 1.9 when 1-pentanesulfonate (2.5 m*M*) was used as counter-ion and 2.3 when 1-decanesulfonate (2.5 m*M*) was chosen as ion-pairing agent.

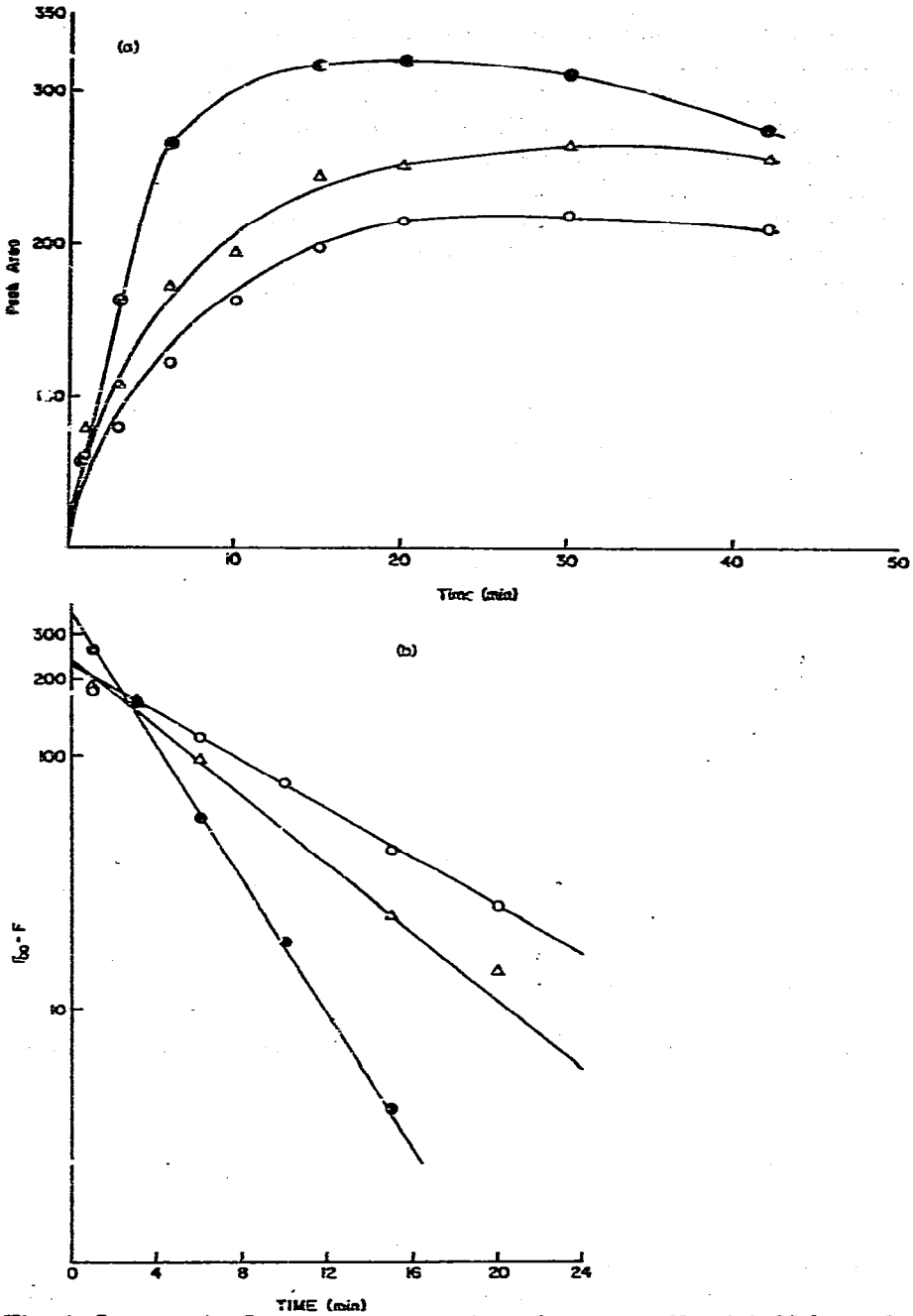


Fig. 1. Increase in fluorescence occurring when tamoxifen (△), N-desmethyltamoxifen (○) and 4-hydroxytamoxifen (●), extracted from plasma, are irradiated with UV light (distance between light and reaction vessel = 10 cm). Reaction carried out as described in the text. Data presented (a) on cartesian coordinates and (b) as a semi-logarithmic plot.

Separation of analytes with maximum resolution from each other and from co-extracted contaminants was obtained using a mobile phase of methanol—water (73:27) containing 0.5% acetic acid and sodium pentanesulfonate (2.5

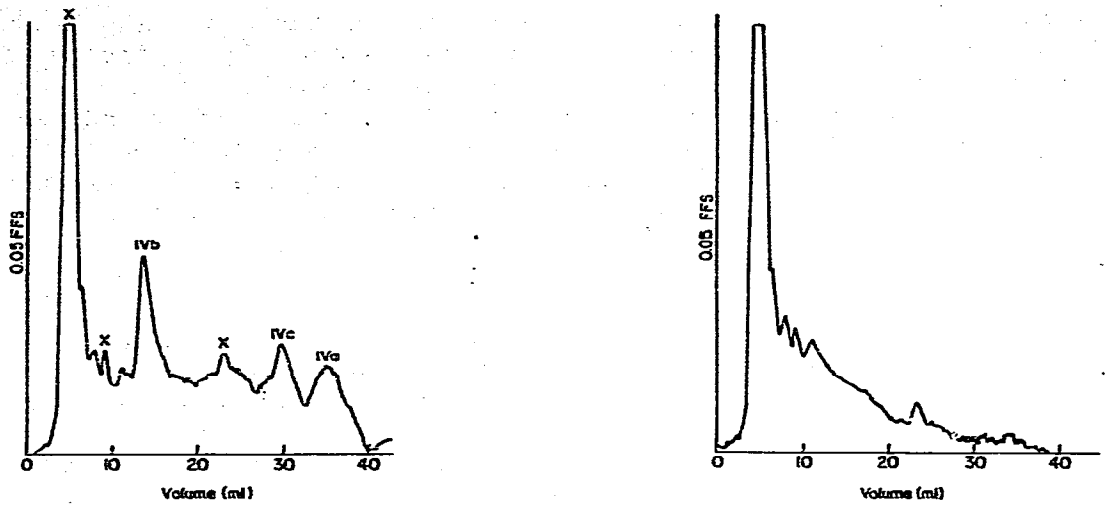


Fig. 2. Chromatography of tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen as their phenanthrene derivatives (IVa—c) obtained by extraction of I, II and III from plasma (each component present at a concentration of 1 ng/ml) and irradiation with UV light (as described in the text). Components were separated by paired-ion reversed-phase (C_{18}) partition chromatography using methanol—water (73:27) containing 0.5% acetic acid and 2.5 m*M* sodium pentanesulfonate as mobile phase. Flow-rate = 2 ml/min. Peaks at V_R = 7 and 22 ml (X) represent co-extracted contaminants also found in plasma blanks.

Fig. 3. A plasma blank containing none of the drug species, but carried through the analytical sequence.

m*M*) (Fig. 2). The retention volumes (V_r) for the analytes are as follows: I, 26 ml; II, 12 ml; III, 24 ml; IVa, 34 ml; IVb, 13.6 ml; IVc, 27.2 ml. Under these conditions no interference was observed from extraneous peaks seen in plasma blanks (Fig. 3).

Components were quantitated as IV by measuring peak area, which was linearly related to concentration of I, II or III in the range 0.1—500 ng/ml of plasma. A standard curve constructed after analysis of plasma samples containing known amounts of I, II and III over this concentration range resulted in the line $y = 0.17x + 3.44$ (correlation coefficient: 0.999) for tamoxifen (monitored as IVa) when subjected to linear regression analysis, the line $y = 0.16x + 1.44$ (correlation coefficient: 0.999) for 4-hydroxytamoxifen (as IVb) and the line $y = 0.21x + 4.99$ (correlation coefficient: 0.998) for N-desmethyltamoxifen (as IVc). Less than 4% variation in these curves was observed as day-to-day variation in the response factor. Over this concentration range, when samples were subjected to the analysis scheme described here, overall analytical recovery of tamoxifen from plasma was 89 ± 4% (n = 9), recovery of II was 97 ± 4% (n = 9) and recovery of III was 83 ± 4% (n = 9). The detection limits for I, II and III are 0.1 ng/ml of plasma at the 99% confidence level as measured as their fluorescent derivatives and as determined by direct analysis of drug-supplemented plasma samples at 100 pg/ml vs. plasma blanks.

This method provides suitable sensitivity to monitor therapeutic levels of

tamoxifen [8, 10] for at least 24 h after oral administration of the drug (dose: 20 mg, daily). 4-Hydroxytamoxifen is a minor metabolite [7] and appears at levels ≤ 20 ng/ml. The major metabolite, III, was detected (< 10 ng/ml) 1 h after the initial dose and reached steady-state levels of 250—350 ng/ml after approximately 40 doses (3 weeks). The sensitivity of the method permits the monitoring of the drug and two of its metabolites for long periods of time and offers an opportunity for also following subtherapeutic amounts of drug.

DISCUSSION

Tamoxifen and its metabolites II and III can be conveniently and efficiently removed from biological fluid by a single extraction with diethyl ether. The difficulty suggested by other groups [7] in detecting appreciable levels of II is apparently due to its facile conversion to IVb during the extraction and, therefore, would only be observed when the methodology dictates first monitoring II as the phenanthrene, IVb, and secondly, forming this derivative prior to the chromatographic step.

The increased stability of IVa—c observed in acid solution may be caused by protonation of the amine function, eliminating the nucleophilic character of the nitrogen. We previously showed [6] that decomposition of IV to less fluorescent products involves loss of the aminoalkanol side chain, which could be initiated by intramolecular nucleophilic attack by the amine nitrogen. Depression of nucleophilicity would thus increase stability.

Chromatographic separation of IVa—c could not be accomplished by either reversed-phase or normal-phase chromatography because resolution of peaks arising from IVb and IVc could not be achieved. Paired ion chromatography with a reversed-phase (C_{18}) column provided resolution of all peaks of interest. The small effect observed in k' in varying the length of the alkyl chain on the hetaeron from C_5 to C_{10} is most likely due to the inherent hydrophobicity of IVa—c and, therefore, partitioning of the ion-pair is only minimally affected by the chain length of the alkylsulfonate.

This tamoxifen assay has the following advantages over the previously reported procedure [6]: (1) it allows simultaneous and selective monitoring of I, II and III; (2) the photolysis products (IVa—c) are more stable in the reaction media; (3) a cleaner chromatogram is produced because photolysis is carried out in the mobile phase and (4) sensitivity is improved by a factor of ca. 20. The method is currently being used to monitor tamoxifen and its 4-hydroxy and desmethyl metabolites in the plasma of women who have been administered the drug in the management of breast cancer.

ACKNOWLEDGEMENTS

This work was supported in part by training grant CA-09242 awarded by the National Cancer Institute, National Institutes of Health (PHS, DHEW) and a grant from Imperial Chemical Industries, Americas.

REFERENCES

1 D.C. Tormey, R.M. Simon, M.E. Lippman, J.M. Bull and C.E. Myers, Cancer Treat. Rep., 60 (1976) 1451.
2 A. Manni, J. Triajillo, J.S. Marshall and O.H. Pearson, Cancer Treat. Rep., 60 (1976) 1445.
3 L.R. Morgan, P.S. Schein, P.V. Woolley, D. Hoth, J. Macdonald, M. Lippman, L.E. Posey and R.W. Beazley, Cancer Treat. Rep., 60 (1976) 1437.
4 M.M. Roberts, A.P.M. Forrest, T. Hamilton, A.O. Langlands, W. Lutz, I.J. McFadyen and H.J. Steward, Cancer Treat. Rep., 60 (1976) 1431.
5 H.J. Lerner, P.R. Bano, L. Israel and B.S. Leving, Cancer Treat. Rep., 60 (1976) 1431.
6 D.W. Mendenhall, H. Kobayashi, F.M.L. Shih, L.A. Sternson, T. Higuchi and C. Fabian, Clin. Chem., 24 (1978) 1518.
7 H.K. Adam, E.J. Douglas and J.V. Kemp, Biochem. Pharmacol., 28 (1979) 145.
8 J.M. Fromson, S. Pearson and V. Bramah, Xenobiotica, 3 (1973) 693, 711.
9 K. Thakker, T. Higuchi and L.A. Sternson, J. Pharm. Sci., 63 (1979) 93.
10 C.J. Fabian, L.A. Sternson, M. El-Serafi, M. Barnett, L. Cain and H. Shih, Cancer, in press.