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ELECTROCHEMICAL DETECTION OF MERCAPTURIC ACID DERIVATIVES AFTER SEPARATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Mercapturic acid derivatives were separated by isocratic elution on a high-performance liquid chromatography column (Inertsil ODS) and detected electrochemically at various applied potentials ($+0.60$–$+0.85$ V). The responses of the electrochemical detector gradually increased with increasing potential but a plateau value was not obtained for all samples tested. When methanol was employed as a modifier of the eluents instead of acetonitrile, the response curves (current *vs.* voltage) were shifted to higher voltages for all mercapturic acid derivatives. Both the linearity ($r > 0.998$) and the reproducibility (coefficient of variation $< 3.5\%$) of the detector response were consistent. The strength of the detector response depended on the atomic configuration near the sulphur atom in the mercapturic acid derivatives. The detection limits of eleven mercapturic acid derivatives at $+0.80$ V were in the range 0.21 [S-(3-hydroxybutyl)mercapturic acid] to 8.3 pmol [S-(2-O-acetylbutyl)mercapturic acid methyl ester].

INTRODUCTION

Baumann and Preusse[1] and Jaffe[2] demonstrated in 1879 that certain sulphur-containing compounds appeared in dog urine after administration of halogenated aromatic compounds like bromobenzene. These compounds, identified as N-acetyl-cysteine conjugates, were called mercapturic acids. There have since been a number of reports concerning the detection of mercapturic acids, many of which involved non-selective methods[3–7]. In the simplest assay procedure, the concentration of thiols, formed by alkaline hydrolysis of the thioether bond, is measured indirectly after their reaction with dithiobis(nitrobenzoic acid) to form 2-nitrobenzoic acid (yellow dye)[8]. This has been used to determine thioether compounds including mercapturic acids in urine samples[3–5]. High background values and large variations in individual values were found in all these studies, however. These were mainly due to high urinary concentrations of endogenous sulphur-containing compounds such as cysteine and cystine. Therefore, selective methods allowing a more specific detection of the mercapturic acid metabolites are needed. A gas chromatography–mass spectrometry

(GC–MS) method which includes the derivatization of the COOH group with an alkylating agent, *e.g.*, diazomethane was recently developed which gave a selective and sensitive detection for this type of compounds in urine[9–13]. Numerous mercapturic acids have since been isolated from biological samples by use of this method, *e.g.*, ethylmercapturic acid[14] and 3-oxobutyl- and 3-hydroxybutylmercapturic acid[15] have been identified from rat urine after administration of urethane and tributylphosphate, respectively.

This paper reports attempts to separate and detect some mercapturic acid derivatives by high-performance liquid chromatography (HPLC) and electrochemical detection (ED). The aim of the research was the development of a simple, selective and sensitive detection method.

EXPERIMENTAL

Materials

S-Ethylmercapturic acid (a in Table I) and S-ethoxycarbonylmercapturic acid (m) were prepared by the method of Boyland and Nery[14]. Other mercapturic acid (MA) derivatives (b–l in Table I) were synthesized according to previous reports[15,16]. Phosphoric acid was of biochemical reagent grade (Wako Pure Chemicals, Osaka, Japan). Water, acetonitrile and methanol were of HPLC grade (Wako Pure Chemicals). All other chemicals were of analytical reagent grade and were used without further purification.

HPLC and ED

The HPLC–ED system is shown schematically in Fig. 1. A Shimadzu (Kyoto, Japan) LC-6A high-performance liquid chromatograph equipped with a Rheodyne injector (Model 7125; Cotati, CA, U.S.A.) was used. The signal from the electro-

TABLE I

STRUCTURES OF MERCAPTURIC ACID DERIVATIVES TESTED

$$R^1 - S - CH_2CH \underset{NHCOCH_3}{\overset{COOR^2}{<}}$$
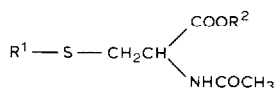
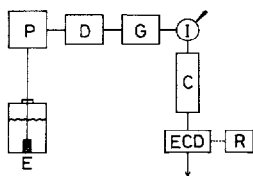|	Derivative	R^1	R^2
a	S-Ethylmercapturic acid	CH_2CH_3	H
b	S-Butylmercapturic acid	$CH_2CH_2CH_2CH_3$	H
c	S-(3-Hydroxybutyl)mercapturic acid	$CH_2CH_2CH(OH)CH_3$	H
d	S-(2-Oxobutyl)mercapturic acid	$CH_2COCH_2CH_3$	H
e	S-(3-Hydroxybutyl)mercapturic acid methyl ester	$CH_2CH_2CH(OH)CH_3$	CH_3
f	S-(3-Oxobutyl)mercapturic acid methyl ester	$CH_2CH_2COCH_3$	CH_3
g	S-(2-Hydroxybutyl)mercapturic acid methyl ester	$CH_2CH(OH)CH_2CH_3$	CH_3
h	S-(2-Oxobutyl)mercapturic acid methyl ester	$CH_2COCH_2CH_3$	CH_3
i	S-*sec.*-Butylmercapturic acid methyl ester	$CH(CH_3)CH_2CH_3$	CH_3
j	S-(4-O-Acetylbutyl)mercapturic acid methyl ester	$CH_2CH_2CH_2CH_2OCOCH_3$	CH_3
k	S-(3-O-Acetylbutyl)mercapturic acid methyl ester	$CH_2CH_2CH(OCOCH_3)CH_3$	CH_3
l	S-(2-O-Acetylbutyl)mercapturic acid methyl ester	$CH_2CH(OCOCH_3)CH_2CH_3$	CH_3
m	S-Ethoxycarbonylic mercapturic acid	$COOCH_2CH_3$	H

Fig. 1. Schematic diagram of the HPLC-ED system for mercapturic acid derivatives. E = Eluent reservoir; P = pump; D = damper; G = guard cell; I = injector; C = analytical column; ECD = coulochemical detector; R = recorder.

chemical detector was recorded on a C-R6A Chromatopac (Shimadzu). An analytical column, Inertsil ODS (150 mm × 4.6 mm I.D., 5 μm) (Gaskuro Kogyo, Tokyo, Japan), was maintained at 40°C in a column oven (Gaskuro Kogyo). The electrochemical cell (Model 5010) of a coulometric detector (Model 5100A; ESA, Bedford, MA, U.S.A.) was placed after the analytical column. The guard cell (Model 5020) of the detector was placed between the pump and the injector. In a separate experiment, a Shimadzu SPD-6A UV spectrophotometer equipped with an 8-μl flow cell was used for the detection of the eluates from the column at 210 nm. All the eluents used as mobile phases were degassed with an ultrasonicator prior to use. The flow-rate of the eluent was 1.0 ml/min.

Sample preparation
A 4 mM stock solution of each mercapturic acid derivative in methanol was prepared. The various concentrations (1.0–10 μM) of MA derivatives were prepared by dilution in water. A fixed volume of the mixed solution was injected into the HPLC column.

RESULTS AND DISCUSSION

HPLC separation of mercapturic acid (MA) derivatives
HPLC separation of MA derivatives was performed on a reversed-phase column before ED. The effect of both the acetonitrile concentration and pH in the eluents on the separation was investigated by isocratic elution on an Inertsil ODS column. Since the MA derivatives tested have hydrophilic COOH and OH groups, many peaks corresponding to the MA derivatives (a–i) were eluted near the void volume in neutral and alkaline solutions (pH 4–8), and complete separation was difficult (data not shown). The separation was gradually improved with decreasing pH (4 to 2), and was efficient at pH 2.2 with 0.15 M H$_3$PO$_4$. The capacity factor, k', of each compound, however, increased as the concentration of acetonitrile decreased (Table II). Effective separation was achieved by isocratic elution with CH$_3$CN–0.15 M H$_3$PO$_4$ (15:85) (eluent I). However, 31 min were required for the elution of the hydrophobic MA methyl esters, *e.g.*, i and l. To overcome this disadvantage, the separation was also attempted with various concentrations of methanol in 0.15 M H$_3$PO$_4$. At the same modifier concentrations, elutions with solvents containing methanol were slower than those with acetonitrile. A complete separation in a short time was impossible by isocratic elution in all eluents containing methanol. Although complete separation in a relatively short time may be provided by gradient or step-

TABLE II

CAPACITY FACTORS, k', OF MERCAPTURIC ACID DERIVATIVES UNDER VARIOUS CONDITIONS

ND = Not determined; UV detection at 210 nm; $k' = t - t_0/t_0$ = 2.0 min. The void volume of the column was measured with acetone as a marker, eluted with 100% methanol.
Eluents: I CH_3CN–0.15 M H_3PO_4 (15:85); II CH_3CN–0.15 M H_3PO_4 (13:87); III CH_3CN–0.15 M H_3PO_4 (10:90); IV CH_3CN–0.15 M H_3PO_4 (8:92); V CH_3CN–0.15 M H_3PO_4 (20:80); VI CH_3CN–0.15 M H_3PO_4 (18:82); VII CH_3CN–0.15 M H_3PO_4 (17:83); VIII CH_3OH–0.15 M H_3PO_4 (20:80); IX CH_3OH–0.15 M H_3PO_4 (18:82); X CH_3OH–0.15 M H_3PO_4 (33:67); XI CH_3OH–0.15 M H_3PO_4 (35:65); XII CH_3OH–0.15 M H_3PO_4 (40:60); XIII CH_3OH–0.15 M H_3PO_4 (45:55); XIV CH_3OH–0.15 M H_3PO_4 (50:50).

MA	k'													
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
c	0.61	0.80	1.39	2.04	ND	ND	ND	1.60	1.93	ND	ND	ND	ND	ND
a	1.03	1.33	1.95	2.75	ND	ND	ND	2.05	2.35	ND	ND	ND	ND	ND
d	1.11	1.44	2.25	3.11	ND	ND	ND	1.79	2.13	ND	ND	ND	ND	ND
e	1.44	1.97	3.46	4.98	ND	ND	ND	3.41	4.17	ND	ND	ND	ND	ND
f	1.65	2.14	3.46	4.98	ND	ND	ND	2.55	3.10	ND	ND	ND	ND	ND
g	1.95	2.63	4.53	6.71	ND	ND	ND	4.33	5.30	ND	ND	ND	ND	ND
h	2.53	3.31	5.33	7.51	ND	ND	ND	3.71	4.52	ND	ND	ND	ND	ND
b	7.92	11.1	ND	ND	3.87	5.06	5.85	ND	ND	5.39	4.56	2.99	1.94	1.35
j	9.27	13.7	ND	ND	4.20	5.59	6.56	ND	ND	4.54	3.71	2.25	1.43	0.94
k	9.78	14.4	ND	ND	4.52	5.99	7.01	ND	ND	4.72	3.89	2.37	1.50	0.94
l	13.0	19.3	ND	ND	5.92	7.92	9.29	ND	ND	6.11	4.99	2.99	1.94	1.35
i	14.5	19.2	ND	ND	6.96	9.02	10.39	ND	ND	7.52	6.29	4.04	2.64	1.80

wise elution, the solvent exchange during an analysis with ED causes a large difference in background current. Therefore, ED of the MA derivatives was carried out in three groups (group A: a, c and f; B: d, e, g and h; C: b, i–l) according to the elution times.

ED of MA derivatives

A coulometric electrochemical cell (Model 5010) attached to a Coulochem 5100A (ESA) has dual series working electrodes made of porous graphite. The guard cell (Model 5020) placed between the pump and the injector pre-oxidizes the mobile phase, reducing the background currents at the working electrodes. For example, with the guard cell operating at +0.85 V, the background current detected with the second electrode (+0.80 V) dropped to 18.1 μA, a value which was 67.7% of that obtained with the guard cell off [eluent used, CH_3CN–0.15 M H_3PO_4 (2:8)]. The guard cell has no effect on the electrochemical oxidation of MA derivatives. However, the eluent may be oxidized by the cell judging from the rising baseline level at 210 nm with increasing potential.

The electrochemical responses obtained with each injection of MA derivatives were plotted against the potential difference applied to the working electrode (second or down-flow electrode). The potential of the first (or up-flow) electrode was fixed at +0.60 V, because all the MA derivatives gave no response at this potential. In the analysis of biological samples, e.g., urine, the use of the first electrode seems to be advantageous for the removal of endogenous compounds oxidized at potentials lower than +0.60 V.

As depicted in Fig. 2, the ED responses increased exponentially with applied potential, however a current plateau was not obtained at all the points tested (+ 0.64–0.85 V). Although the use of potentials higher than + 0.85 V may result in high currents, the background current based on the eluents must also increase, as judged by the results shown in Fig. 3. In addition, the elevated potentials will reduce the lifetime of the cell and so applied potentials higher than + 0.85 V were not examined in all the experiments.

When CH_3OH–0.15 M H_3PO_4 (18:82) was used as the eluent, the currents at certain potentials were lower than those in CH_3CN–0.15 M H_3PO_4 (10:90), and the current–voltage (C–V) curves shifted to higher voltages for all MA derivatives tested (Fig. 2C). The currents for compounds a and c (Fig. 2C) in CH_3OH–0.15 M H_3PO_4 (18:82) were almost the same at all voltages, in contrast to the results obtained in CH_3CN–0.15 M H_3PO_4 (10:90) (Fig. 2A). The background currents were reduced compared with those in CH_3CN–0.15 M H_3PO_4 (20:80) (Fig. 3). Since the response in the eluent containing acetonitrile was higher than those in methanol at the same voltage, acetonitrile was selected as the modifier for ED of MA derivatives in subsequent experiments.

The ED response to various concentrations of MA derivatives injected was also determined. As shown in Fig. 4, different concentrations gave a linear current passing through the origin ($r > 0.998$). The coefficient of variation (C.V.) of the ED response at + 0.80 V was tested at two different concentrations (50 and 250 pmol) and was reproducible and lower than 3.5% (Table III).

The C vs. V curves in Fig. 2A and B indicate that the derivatives having COOH groups (a–c) but not d were relatively sensitive to electrochemical oxidation. The effect of the substituent groups –OH and –C = O at the same position was not obvious from a comparison between compounds e and f or between g and h. The existence of certain groups adjacent to the sulphur atom reduced the electrochemical responses (g and h against e and f). Therefore, the ED responses seem to depend on the distance of the particular group from the sulphur atom. A similar phenomenon was also ob-
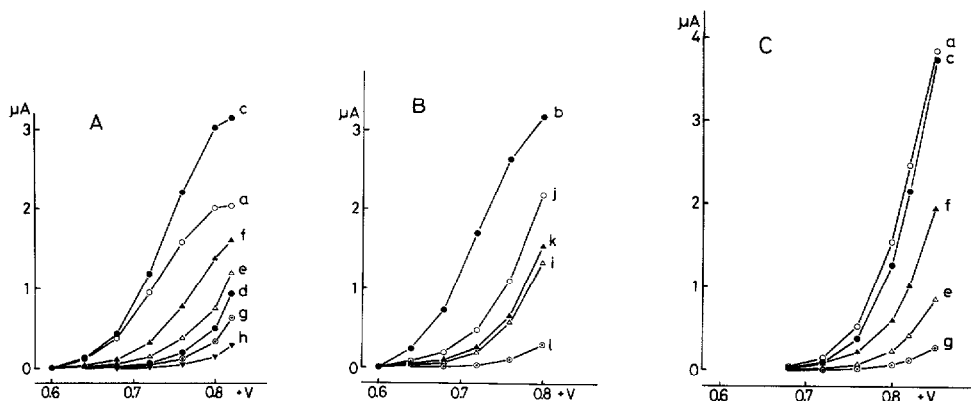

Fig. 2. Current vs. voltage (C vs. V) curves for mercapturic acid derivatives. Compounds a–l in Table I. Up-flow potential: + 0.60 V. Guard cell potential: + 0.85 V. (A) CH_3CN–0.15 M H_3PO_4 (10:90) (eluent III); (B) CH_3CN–0.15 M H_3PO_4 (20:80) (eluent V); (C) CH_3OH–0.15 M H_3PO_4 (18:82) (eluent IX). Other HPLC conditions as in Experimental section.
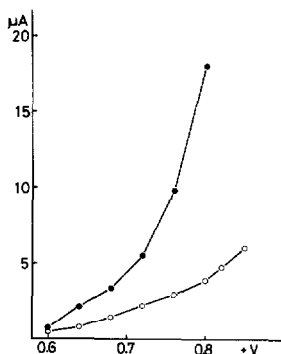
Fig. 3. Change of background current with applied potential. ●-●, CH_3CN–0.15 M H_3PO_4 (20:80) (eluent V); ○-○, CH_3OH–0.15 M H_3PO_4 (18:22) (eluent IX). HPLC and ED conditions as in Fig. 2.

served by comparison of O-acetyl esters (order of response, j > k > l). Steric hindrance also decreased the ED response (i in Fig. 2). On the other hand, S-ethoxycarbonylmercapturic acid (m), having both an amide group ($NHCOCH_3$) and an ethoxycarbonyl group (S–$COOCH_2CH_3$), showed no response to ED at any potential (+ 0.60–0.85 V). The above data suggest that the amide group is not responsible for the electrochemical oxidation, but the sulphur is oxidized by the electrochemical cell.
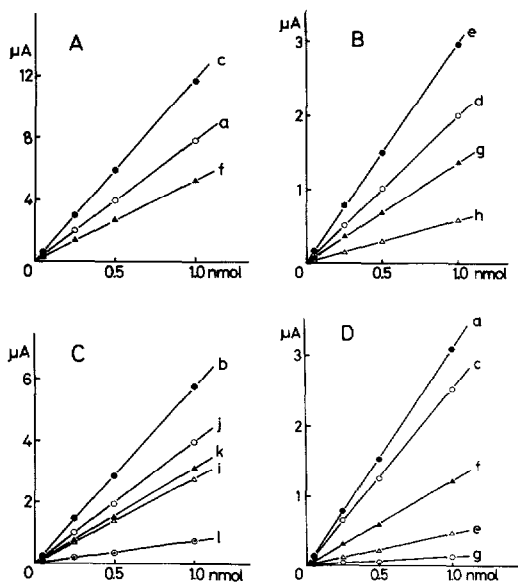

Fig. 4. Linearity of electrochemical response for various amounts of mercapturic acid derivatives. Up-flow potential: + 0.60 V. Down-flow potential: + 0.80 V. Guard cell potential: + 0.85 V. (A) and (B) CH_3CN–0.15 M H_3PO_4 (10:90) (eluent III); (C) CH_3CN–0.15 M H_3PO_4 (20:80) (eluent V); (D) CH_3OH–0.15 M H_3PO_4 (18:82) (eluent IX). Other HPLC conditions as in Experimental section.

TABLE III

REPRODUCIBILITY OF ED RESPONSE

Eluents as in Table II. HPLC and ED conditions as in Fig. 4.

MA	Eluent	C.V. (%), n = 5	
		50 pmol injected	250 pmol injected
c	III	2.1	0.25
a	III	1.8	0.14
f	III	1.8	0.21
d	III	3.5	0.40
e	III	2.6	0.45
g	III	3.4	1.8
b	V	1.3	1.2
j	V	1.8	1.9
k	V	2.1	2.0
l	V	1.4	1.8
i	V	3.2	2.9

Typical chromatograms obtained with both UV detection at 210 nm and ED at + 0.80 V in eluents III and V are depicted in Fig. 5. At 210 nm, similar peak heights for various MA derivatives were obtained in each chromatogram, whereas the ED responses at + 0.80 V were different for the individual MA derivatives. The detection limits for the derivatives under the proposed conditions were estimated as 0.21–8.3 pmol, lower than those with UV detection at 210 nm (1.2–3.9 pmol) except for compounds h and l (Table IV). The detection limits may be improved by recycling of the eluents which may make the baseline more stable, and by a decrease in phosphoric acid concentration in the eluents in order to reduce the background current.
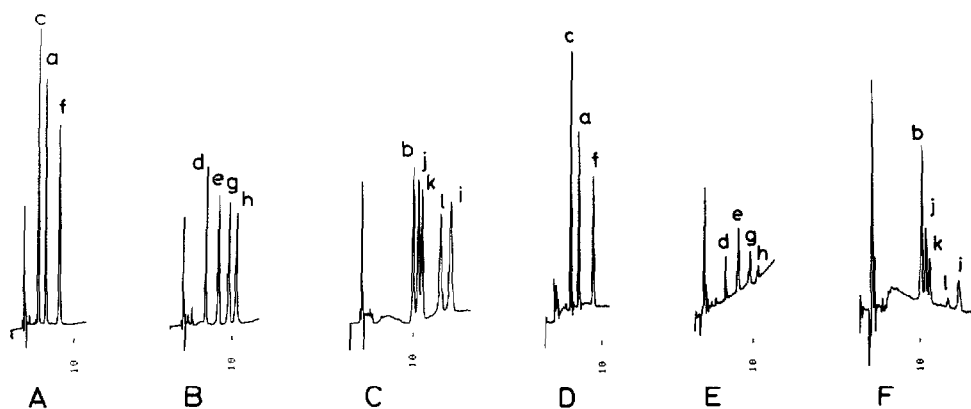

Fig. 5. Chromatograms of mercapturic acid derivatives obtained by UV and ED detections. (A)–(C) UV detection at 210 nm (250 pmol each); (D)–(F) ED at + 0.80 V (up-flow potential, + 0.60 V; guard cell potential, + 0.85 V) (25 pmol each). (A), (B), (D) and (E) $CH_3CN–0.15$ M H_3PO_4 (10:90) (eluent III); (C) and (F) $CH_3CN–0.15$ M H_3PO_4 (20:80) (eluent V). Other HPLC conditions as in Experimental section.

TABLE IV

COMPARISON OF DETECTION LIMITS BETWEEN UV AND ED

ND = Not determined. Eluents as in Table II. HPLC and ED conditions as in Fig. 4.

MA	Detection limit (S/N = 2) (pmol)			
	UV (210 nm)		ED (+0.80 V)	
	III	V	III	V
c	1.2	ND	0.21	ND
a	1.4	ND	0.31	ND
f	1.8	ND	0.42	ND
d	2.7	ND	1.2	ND
e	3.3	ND	0.82	ND
g	3.5	ND	1.7	ND
h	3.9	ND	4.0	ND
b	ND	2.6	ND	0.47
j	ND	2.9	ND	1.1
k	ND	3.1	ND	1.8
i	ND	3.1	ND	2.4
l	ND	3.6	ND	8.3

Most mercapturic acids have been identified as urinary metabolites originating from xenobiotic compounds[17]. Therefore, the mercapturic acid assay will help to assess human exposure to harmful chemicals, if there is a quantitative relationship between the values in urine and the degree of exposure. Generally speaking, when the urinary MA excretion is extremely low, the assay is difficult due to interference from endogenous compounds. The proposed method in combination with HPLC and ED is sensitive enough (Table IV). Furthermore, the method is simple and does not require the derivatization used in GC methods. Therefore, with a suitable pretreatment, the HPLC–ED method may be useful for the routine analysis of MAs in biological samples. The application of this method is currently being evaluated and the results will be reported elsewhere.

REFERENCES

1 E. Baumann and C. Preusse, Ber. Dtsch. Chem. Ges., 12 (1879) 806–810.
2 M. Jaffe, Ber. Dtsch. Chem. Ges., 12 (1879) 1092–1098.
3 F. Buffoni, G. Santoni, V. Albanese and P. Dolara, J. Appl. Toxicol., 3 (1983) 63–65.
4 F. Seutter-Berlage, H. L. van Dorp, H. G. J. Kosse and P. T. Henderson, Int. Arch. Occup. Environ. Health, 39 (1977) 45–51.

5 H. Vainio, H. Savolainen and I. Kilpikari, *Br. J. Ind. Med.*, 35 (1978) 232–234.
6 K. H. Summer, K. Rozman, F. Coulston and H. Greim, *Toxicol. Appl. Pharmacol.*, 50 (1979) 207–212.
7 R. van Doorn, R. P. Bos, R. M. E. Brouns, C.-M. Leijdekkers and P. T. Henderson, *Arch. Toxicol.*, 43 (1980) 293–304.
8 G. L. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70–77.
9 T. Seutter-Berlage, L. P. C. Delbressine, F. L. M. Smeets and H. C. J. Ketelaars, *Xenobiotica*, 8 (1978) 413–418.
10 P. J. van Bladeren, D. D. Breimer, G. M. T. Rotteveel-Smijs, R. A. W. de Jong, W. Buijs, A. van der Gen and G. R. Mohn, *Biochem. Pharmacol.*, 29 (1980) 2975–2982.
11 J. D. Osterloh, B.-S. Cohen, W. Popendorf and S. M. Pond, *Arch. Environ. Health*, 39 (1984) 271–275.
12 W. Dekant, M. Metzler and D. Henschlar, *Biochem. Pharmacol.*, 35 (1986) 2455–2458.
13 W. Onkenhout, P. P. J. Mulder, P. J. Boogaard, W. Buijs and N. P. E. Vermeulen, *Arch. Toxicol.*, 59 (1986) 235–241.
14 E. Boyland and R. Nery, *Biochem. J.*, 94 (1965) 198–208.
15 T. Suzuki, K. Sasaki, M. Takeda and M. Uchiyama, *J. Agric. Food Chem.*, 32 (1984) 1278–1283.
16 T. Suzuki, K. Sasaki, M. Takeda and M. Uchiyama, *J. Agric. Food Chem.*, 32 (1984) 603–610.
17 L. F. Chasseaud, *Adv. Cancer Res.*, 29 (1979) 175–275.