Journal of Chromatography, 419 (1987) 17–25
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3722

SENSITIVE FERROCENE REAGENTS FOR DERIVATIZATION OF THIOL COMPOUNDS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DUAL-ELECTRODE COULOMETRIC DETECTION

KAZUTAKE SHIMADA, TOMOYUKI OE and TOSHIO NAMBARA*

Pharmaceutical Institute, Tohoku University, Sendai 980 (Japan)

(First received February 16th, 1987; revised manuscript received March 23rd, 1987)

SUMMARY

Three N-substituted maleimides possessing ferrocene as an electrophore were prepared and evaluated for pre-column derivatization of thiol compounds in high-performance liquid chromatography with dual-electrode coulometric detection. The utility of these reagents was investigated by employing N-acetyl-L-cysteine as a model compound. Among the three, N-(ferrocenyl)maleimide was the most favourable reagent with respect to reactivity, stability and electrochemical properties. The dual-electrode coulometric detection of the adduct showed high selectivity and sensitivity with a detection limit of 0.06 pmol. The proposed method is applicable to the determination of glutathione in biological specimens.

INTRODUCTION

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) is a useful method for the determination of trace components in complex matrices because of its excellent selectivity and sensitivity [1]. In recent years, various pre- and post-column labelling methods have been developed to extend its applicability [2–7]. In previous studies in this series, we proposed novel ferrocene reagents for the pre-column labelling of amines [3], hydroxy compounds [5], carboxylic acids [6] and glucuronides [7] in HPLC–ED. As the ferrocene derivative undergoes facile oxidation and the product is in turn readily reduced, it can be detected selectively in the presence of other electroactive compounds, such as phenols, catechols and aromatic amines.

This paper deals with the preparation and properties of derivatization reagents having ferrocene as an electrophore for the determination of thiol compounds by HPLC–ED. In addition, the application of this method to the determination of glutathione in biological specimens is described.

0378-4347/87/$03.50

EXPERIMENTAL

Materials

(Dimethylaminomethyl)ferrocene was purchased from Aldrich (Milwaukee, WI, U.S.A.). Ferrocenecarboxylic acid (Ia) and 1,1′-dimethylferrocene were supplied by Tokyo Kasei Kogyo (Tokyo, Japan), the latter being purified by chromatography on Toyo Pak ODS (Toyo Soda, Tokyo, Japan) to remove contaminating monomethylferrocene. 2-Hydroxyoestrone (2-OHE$_1$) was prepared by the method reported by Stubenrauch and Knuppen [8]. N-(4-Dimethylaminophenyl)maleimide was synthesized in our laboratories [9]. All other reagents and chemicals were purified by recrystallization or distillation prior to use.

Silica gel 60 and silica gel HF$_{254}$ (E. Merck, Darmstadt, F.R.G.) were used for column chromatography and thin-layer chromatography (TLC), respectively.

Instruments

HPLC was carried out on a Waters 510 chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with an ESA 5100A coulometric detector (Environmental Sciences Assoc., Bedford, MA, U.S.A.). ESA 5020 and 5011 cells (Environmental Sciences Assoc.) were used as guard and dual electrode analytical cells with a porous graphite electrode, respectively. The applied potential was set versus a palladium reference electrode. A YMC-GEL C$_8$ (5 μm) column (15 cm×0.4 cm I.D.) (Yamamura Chem. Lab., Kyoto, Japan) was used at a flow-rate of 1 ml/min at ambient temperature.

Preparation of derivatization reagents

Melting points were measured on a micro hot-stage apparatus (Yanagimoto, Kyoto, Japan) and were uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on JEOL (Tokyo, Japan) FX-90A, FX-100 and GX-500 at 90, 100 and 500 MHz, respectively, using tetramethylsilane as an internal standard (I.S.). Abbreviations used are s=singlet, t=triplet and m=multiplet. Low- and high-resolution mass spectral (MS) measurements were run on Hitachi (Tokyo, Japan) M-52G and JEOL JMS-01SG-2 spectrometers, respectively.

N-(Ferrocenyl)maleimide (Ig)

Compound Ig was prepared from Ia according to the procedure described by Furdik et al. [10], and was obtained as deep purple prisms [m.p. 151–152°C (from diethyl ether); literature [10] m.p. 135–151°C]. Low-resolution MS: *m/z*: 281 (M^+). ^{1}H NMR (90 MHz) (C^2HCl_3): δ 4.05–4.25 (7H, m, ferrocenyl H), 4.79 (2H, br s, ferrocenyl H), 6.72 (2H, s, vinyl H).

N-(3,1′-Dimethylferrocenyl)maleimide (IIg)

1,1′-Dimethylferrocene-3-carboxylic acid (IIa) was prepared from 1,1′-dimethylferrocene according to the procedure described by Falk et al. [11]. Compound IIa (100 mg) was stirred with phosphorous trichloride (0.8 ml) in dry benzene (3 ml) at 60–70°C for 3 h and then at room temperature overnight. After

evaporation of the solvent, the residue obtained (IIb; 100 mg) was dissolved in dry acetone (0.5 ml), cooled to 0–5 °C and treated with sodium azide (50 mg) in water (0.1 ml). The reaction mixture was allowed to stand at room temperature for 30 min and then extracted with diethyl ether. The organic layer was washed with water, dried over anhydrous sodium sulphate and evaporated to give the acid azide (IIc) as an orange oily substance (90 mg). High-resolution MS: *m/z* 283.0415 (M^+) (calculated for $C_{13}H_{13}FeN_3O$, 283.0409).

A solution of IIc (90 mg) in benzyl alcohol (0.2 ml) was heated gradually and kept at 120 °C for 15 min. The reaction mixture containing the urethane (IId) was diluted with 10% potassium hydroxide solution (2 ml) and refluxed for 4 h under a stream of nitrogen. The reaction mixture was extracted with diethyl ether three times and the combined extracts were washed with 10% hydrochloric acid three times. The extract was rendered strongly alkaline with 10% sodium hydroxide solution and then extracted with diethyl ether. The organic layer was washed with water, dried over anhydrous sodium sulphate and then evaporated to give the amine (IIe) as a red oily substance (15 mg). Low-resolution MS: *m/z* 229 (M^+).

A mixture of IIe (15 mg) and maleic anhydride (10 mg) in chloroform (1 ml) was kept at room temperature overnight. The reaction mixture was applied to a column packed with silica gel 60 (15 cm×0.6 cm I.D.). Elution with benzene–ethyl acetate (1:1) and recrystallization of the dried eluate from diethyl ether–methanol gave N-(3,1′-dimethylferrocenyl)maleamic acid (IIf) as brown-violet plates (15 mg) (m.p. 147–149 °C). High-resolution MS: *m/z* 327.0567 (M^+) (calculated for $C_{16}H_{17}FeNO_3$, 327.0557).

Sodium acetate (60 mg) was added to a solution of IIf (15 mg) in acetic anhydride (1 ml) and heated at 60–80 °C for 4 h. The mixture was poured on to ice–water, stirred for 30 min and then extracted with diethyl ether. The organic layer was washed with 5% sodium hydrogencarbonate and water, dried over anhydrous sodium sulphate and then evaporated. The residue obtained was subjected to preparative TLC using benzene as a developing solvent and the adsorbent corresponding to the spot of R_F 0.47 was eluted with ethyl acetate. Recrystallization of the dried eluate from diethyl ether gave N-(3,1′-dimethylferrocenyl)maleimide (IIg) as dark brown prisms (10 mg; yield 8.3%) (m.p. 76–78 °C). High resolution MS: *m/z* 309.0447 (M^+) (calculated for $C_{16}H_{15}FeNO_2$, 309.0450). ^1H NMR (100 MHz) (C^2HCl_3): δ 1.83 (6H, s, 2×CH_3), 4.06 (5H, br s, ferrocenyl H), 4.80 (2H, br s, ferrocenyl H), 6.66 (2H, s, vinyl H).

N-(2-Ferrocenylethyl)maleimide (IIIg)

2-Ferrocenylethylamine (IIIe) was prepared from (dimethylaminomethyl)ferrocene as reported previously [7,12]. A mixture of IIIe (400 mg) and maleic anhydride (200 mg) in chloroform (2 ml) was kept at room temperature overnight. The reaction mixture was applied to a column packed with silica gel 60 (15 cm×0.6 cm I.D.). Elution with benzene–ethyl acetate (1:1) and recrystallization of the dried eluate from methanol–dichloromethane gave N-(2-ferrocenylethyl)maleamic acid (IIIf) as dark brown plates (400 mg) (m.p.

145–150°C). Calculated for $C_{16}H_{17}FeNO_3$, C 58.74, H 5.24, N 4.28; found, C 58.51, H 5.06, N 4.25%. High-resolution MS: *m/z* 327.0539 (M^+) (calculated for $C_{16}H_{17}FeNO_3$, 327.0556). ^1H NMR (500 MHz) (C^2HCl_3): δ 1.92 (2H, br s, ferrocenyl-CH_2–), 3.35 (2H, br s, –CH_2N), 5.00–5.40 (9H, br s, ferrocenyl H), 6.08 (1H, br s, vinyl H), 6.33 (1H, br s, vinyl H).

Sodium acetate (60 mg) was added to a solution of IIIf (400 mg) in acetic anhydride (1 ml) and heated at 60–80°C for 4 h. The mixture was treated in the manner described for IIg. The crude product was subjected to preparative TLC using benzene as a developing solvent and the adsorbent corresponding to the spot of R_F 0.40 was eluted with ethyl acetate. Recrystallization of the dried eluate from diethyl ether gave N-(2-ferrocenylethyl)maleimide (IIIg) as orange plates (100 mg; yield 18.5%) (m.p. 117–118°C). Calculated for $C_{16}H_{15}FeNO_2$, C 62.16, H 4.89, N 4.53; found, C 62.07, H 4.87, N 4.77%. ^1H NMR (500 MHz) (C^2HCl_3): δ 2.54 (2H, br s, ferrocenyl-CH_2–), 3.64 (2H, t, J=7.4 Hz, –CH_2N), 4.10–4.30 (9H, m, ferrocenyl H), 6.69 (2H, s, vinyl H).

Derivatization of N-acetyl-L-cysteine

Each derivatization reagent (5.5 μg; ca. 3 equiv.) in acetone (0.1 ml) was added to a solution of N-acetyl-L-cysteine (1 μg) in 1/15 *M* phosphate buffer (pH 6.8, 0.4 ml) containing 1 m*M* EDTA and the solution was kept at 0°C in an ice-bath. The reaction was terminated by removal of the excess of reagent by extraction with diethyl ether–hexane (1:1, 3×2 ml). An aliquot of the remaining aqueous layer was subjected to HPLC.

Assay procedure for determination of blood glutathione

Fresh blood, drawn with a syringe from a vein, was diluted 20-fold with 1/15 *M* phosphate buffer under ice cooling. A 0.1-ml aliquot of this sample solution was placed in a tube containing 0.5% Ig solution in acetone (50 μl) and N-acetyl-L-cysteine (I.S., 1.5 μg) in 1/15 *M* phosphate buffer (0.44 ml). The solution was vortex-mixed and allowed to stand at 0°C for 30 min. The excess of reagent was removed by extraction with diethyl ether–hexane (1:1, 3×2 ml) and the aqueous layer was treated with acetone (2 ml). After centrifugation at 1100 *g* for 5 min, an aliquot of the supernatant was subjected to HPLC.

Assay procedure for determination of hepatic glutathione

Male mice weighing 30–40 g were killed and the perfused liver in 1/15 *M* phosphate buffer was boiled for 5 min [13]. The denatured liver tissue was homogenized in 1/15 *M* phosphate buffer, suitably diluted and then subjected to the assay procedure as described above. Protein was measured by the method of Lowry et al. [14] using bovine serum albumin as a reference.

Recovery test for glutathione in biological samples

Fresh mouse liver was homogenized in 1/15 *M* phosphate buffer and kept for several days at 4°C. The resulting homogenate, which contained no glutathione, was heated as described above and then used as a control liver homogenate. The spiked samples were prepared by addition of 1, 2.5 or 4 μg each of glutathione to standard assay tubes containing human blood sample or denatured liver homog-

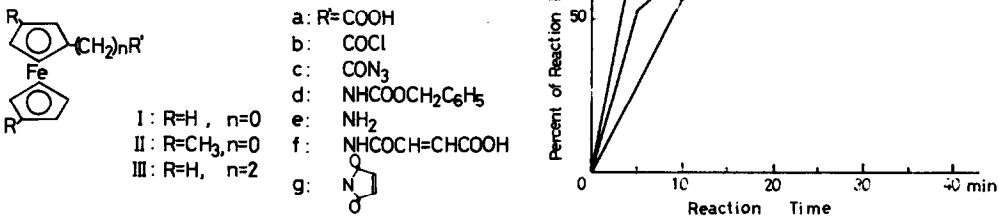

Fig. 1. Structures of derivatization reagents and their synthetic intermediates.

Fig. 2. Time course for derivatization of N-acetyl-L-cysteine. 1, Ig; 2, IIg; 3, IIIg.

enate (0.2 mg of protein per tube). Derivatization with Ig followed by HPLC was carried out in the manner described above.

RESULTS AND DISCUSSION

The design of a promising derivatization reagent for thiol compounds in HPLC–ED requires two structural features, viz., a functional group reactive toward the thiol group and responsive to an electrochemical detector with high sensitivity. In this study, three N-substituted maleimides possessing ferrocene as an electrophore were prepared. N-(Ferrocenyl)maleimide (Ig), N-(3,1′-dimethylferrocenyl)maleimide (IIg) and N-(2-ferrocenylethyl)maleimide (IIIg) were prepared from Ia, 1,1′-dimethylferrocene and (dimethylaminomethyl)ferrocene, respectively. The structures of these reagents and their synthetic intermediates are shown in Fig. 1.

The reactivities of Ig, IIg and IIIg towards the thiol group were examined employing N-acetyl-L-cysteine as a model compound. Reaction of N-acetyl-L-cysteine with the derivatization reagent was performed in phosphate buffer (pH 6.8) at 0°C. The peak heights of the thiol adducts with Ig, IIg and IIIg increased with the reaction time and reached a plateau after 10, 30 and 50 min, respectively (Fig. 2). The adducts with Ig and IIIg were stable whereas that with IIg was decomposed when incubated at 37°C for 30 min.

The electrochemical properties of these adducts were investigated with a dual-electrode coulometric detector with twin electrodes in series. The hydrodynamic voltammograms of these adducts for an anodic response (upstream electrode) are shown in Fig. 3a. The half-wave potentials ($E_{1/2}$) of the adducts with IIg (−60 mV) and IIIg (−80 mV) were lower than that of the adduct with Ig (+30 mV). This can be ascribed to the negative inductive effect of alkyl groups in IIg and IIIg. Among common electroactive compounds, such as phenols (oestriol;

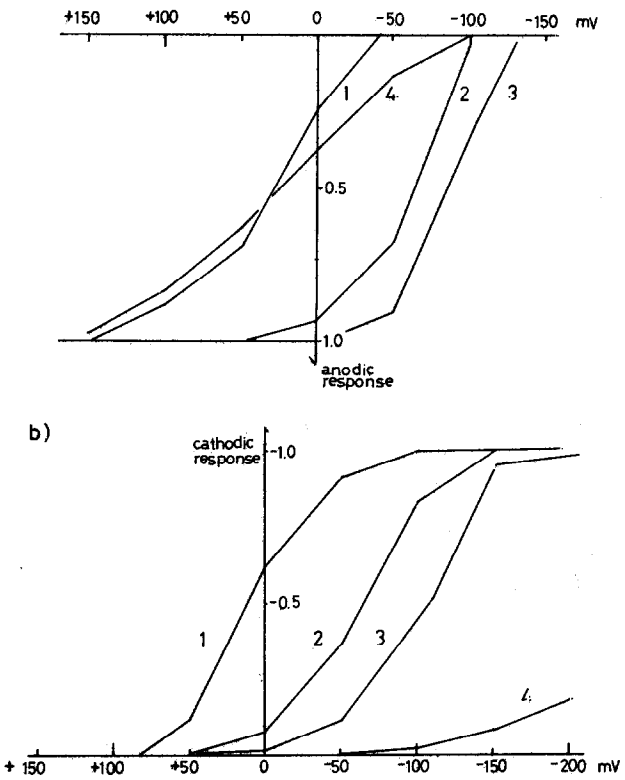

Fig. 3. Hydrodynamic voltammograms of N-acetyl-L-cysteine derivatives and 2-OHE_1. (a) Anodic response; (b) cathodic response. The maximum response of each compound was taken as 1.0. 1, Ig; 2, IIg; 3, IIIg; 4, 2-OHE_1.

$E_{1/2}$ +490 mV), catechol (2-OHE_1; $E_{1/2}$ +25 mV) and aromatic amines [N-(4-dimethylaminophenyl)maleimide-N-acetyl-L-cysteine adduct; $E_{1/2}$ +470 mV], only catechol exhibited an anodic response at the applied potential (+150 mV) where the ferrocene derivative gave the highest response. The oxidation product of the adduct with Ig was reduced at the downstream electrode (−100 mV). In contrast, catechol showed only a feeble response at −100 mV and the maximum cathodic response at −600 mV (Fig. 3b). As a consequence, highly selective and sensitive detection was achieved for the adduct with Ig by the use of the dual-electrode system, the detection limit being 0.06 pmol signal-to-noise ratio=5, 10.1 nA full-scale). It is evident from these data that Ig is the most favourable with respect to reactivity, stability and electrochemical properties.

The utility of Ig was then tested for the determination of glutathione in human blood. On treatment with Ig, glutathione, L-cysteine and N-acetyl-L-cysteine were derivatized at 0°C in 30 min. The resulting adducts were well separated by HPLC on YMC-GEL C_8, as illustrated in Fig. 4a. A whole blood sample was haemolysed and immediately suspended in a tube containing Ig and N-acetyl-L-cysteine (I.S.) in phosphate buffer. It was kept at 0°C for 30 min and the excess of reagent was removed by extraction with diethyl ether–hexane (1:1). The aqueous layer was

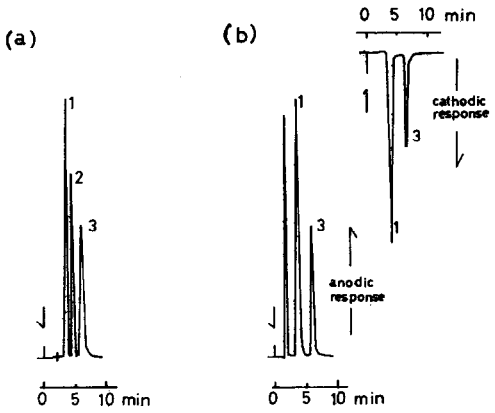

Fig. 4. High-performance liquid chromatograms of glutathione in human blood. (a) Authentic sample; (b) whole blood. Peaks: 1 = glutathione; 2 = L-cysteine; 3 = N-acetyl-L-cysteine (I.S.). Conditions: mobile phase, acetonitrile–0.32% disodium hydrogenphosphate (pH 5.0 adjusted with phosphoric acid) (2:5); guard electrode, +200 mV; upstream electrode, +150 mV; downstream electrode, −100 mV.

deproteinized with acetone and then subjected to HPLC–ED. A typical chromatogram is illustrated in Fig. 4b. Each adduct of glutathione and I.S. exhibited a single peak of the theoretical shape without any interfering peaks. The two peaks were unequivocally identified by comparison with authentic samples with respect to the collection efficiency (the ratio of the current at the downstream detector to that at the upstream detector), the value being 0.76. When the ratio of the amount of glutathione to I.S. (1 μg) was plotted against the peak-height ratio, a linear relationship was observed in the range 0.5–5 μg per tube, the regression equation being $y = 0.44x$. In addition, glutathione added to blood specimens at two levels (1 and 2.5 μg per tube) showed recoveries of $\geqslant 94.1\%$ (coefficient of variation, C.V. $< 5\%$; $n = 9$) (Table I). These results demonstrate that the proposed method is satisfactory with respect to accuracy and precision. The concentrations of glutathione in blood samples taken from six male healthy volunteers

TABLE I

RECOVERY OF GLUTATHIONE ADDED TO HUMAN BLOOD

The recovery of N-acetyl-L-cysteine (1 μg) under the same conditions was $95.1 \pm 1.6\%$ ($n = 10$).

Glutathione (μg per tube)			Recovery (Mean ± S.D., $n = 9$) (%)
Added	Expected	Found	
0	-	0.87	
1.00	1.87	1.85	98.6 ± 4.8
0	-	0.75	
2.50	3.25	3.10	94.1 ± 4.5

TABLE II

BLOOD LEVELS OF GLUTATHIONE IN HEALTHY MALE VOLUNTEERS

Subject	Age (years)	Mean concentration ($n=2$) (μg per 0.1 ml)
K.S.	43	50.9
Y.S.	26	38.8
T.O.	24	48.5
Y.S.	22	37.9
K.S.	22	44.8
T.W.	22	51.3
Mean		45.4
S.D.		5.9

are listed in Table II. These data are in agreement with those reported by Toyo'oka and Imai [15].

The method was further applied to the determination of glutathione in mouse liver. The fresh liver was immediately boiled for 5 min to inactivate unknown factors that would deplete the glutathione level [13]. The denatured liver tissue was homogenized and processed as for blood specimens. Glutathione added to the control liver homogenate at two levels (1 and 4 μg per tube) showed recoveries of $\geqslant 94.5\%$ (C.V. $< 5\%$; $n=10$). It is evident from Fig. 5 that the results obtained by HPLC–ED are in good accord with those given by Ellman's method [16,17]. Pre-column derivatization with Ig appears to be of great use for the determination of picomole levels of thiol compounds by HPLC with dual-electrode coulometric detection. Further applications to the determination of thiol compounds in biological fluids are being conducted and the details will be reported elsewhere.

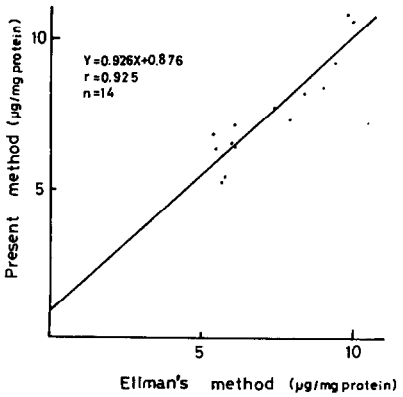

Fig. 5. Correlation of glutathione levels in mouse liver determined by the present method and Ellman's method.

ACKNOWLEDGEMENTS

The authors are indebted to all the staff of the central analytical laboratory of this institute for elemental analyses and spectral measurements. This work was supported, in part, by a grant from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 P.T. Kissinger, Anal. Chem., 49 (1977) 447A.
2 K. Shimada, M. Tanaka and T. Nambara, J. Chromatogr., 280 (1983) 271, and references cited therein.
3 M. Tanaka, K. Shimada and T. Nambara, J. Chromatogr., 292 (1984) 410.
4 I.S. Krull, C.M. Selavka, C. Duda and W. Jacobs, J. Liq. Chromatogr., 8 (1985) 2845.
5 K. Shimada, S. Orii, M. Tanaka and T. Nambara, J. Chromatogr., 352 (1986) 329.
6 K. Shimada, C. Sakayori and T. Nambara, J. Liq. Chromatogr., in press.
7 K. Shimada, E. Nagashima, S. Orii and T. Nambara, J. Pharm. Biomed. Anal., in press.
8 G. Stubenrauch and R. Knuppen, Steroids, 28 (1976) 733.
9 K. Shimada, M. Tanaka and T. Nambara, J. Chromatogr., 227 (1982) 445.
10 M. Furdik, Š. Toma and J. Suchý, Chem. Zvesti, 17 (1963) 21.
11 H. Falk, G. Haller and K. Schlogl, Mh. Chem., 98 (1967) 592.
12 D. Lednicer, J.K. Lindsay and C.R. Hauser, J. Org. Chem., 23 (1958) 653.
13 D.E. Moody, M.H. Silva and B.D. Hammock, Biochem. Pharmacol., 35 (1986) 2073.
14 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
15 T. Toyo'oka and K. Imai, J. Chromatogr., 282 (1983) 495.
16 G.L. Ellman, Arch. Biochem. Biophys., 82 (1959) 70.
17 H.L. Gurtoo, J.H. Hipkens and S.D. Sharma, Cancer Res., 41 (1981) 3584.