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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–FLUOROMETRY FOR THE DETERMINATION OF THIOLS IN BIOLOGICAL SAMPLES USING N-[4-(6-DIMETHYLAMINO-2-BENZOFURANYL)PHENYL]-MALEIMIDE

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SUMMARY

The selective determination of thiols in biological samples was investigated by high-performance liquid chromatography using N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide, which was found to give fluorescent products when treated with certain thiols. Six kinds of thiol (reduced glutathione, cysteine, N-acetylcysteine, cysteamine, homocysteine and coenzyme A) could be separated simultaneously within ca. 12 min and determined at final level of sensitivity. The method was successfully applied to the determination of thiols in rat tissues and plasma and in human normal serum.

INTRODUCTION

In biochemistry, medicine and pharmacology, it is important to determine thiols and related compounds selectively, sensitively and easily. A number of approaches to the assay of thiols are being explored. Recently, a review on the determination of thiols and related compounds using high-performance liquid chromatography (HPLC) was published by Perrett and Rudge [1]. It is well known that a series of non-fluorescent maleimides with aromatic side-chains give rise to strongly fluorescent products when treated with thiols [2–11].

Recently, we synthesized a new fluorogenic reagent, N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM, Fig. 1) and investigated its application to the determination of several thiols and related compounds [12–15]. DBPM is easily prepared from commercially obtainable materials, and both its reactivity with thiols and the fluorescence stability of the reaction products seem to be superior to those of the known maleimide reagents [12,14]. However, selec-

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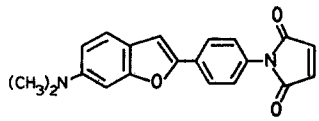

Fig. 1. Chemical structure of DBPM.

tive determination is unfortunately not possible, because the fluorescence wavelengths of the reaction products from the thiols tested are nearly identical.

The present paper describes a selective HPLC method, using DBPM, that enables the assay of a mixture of thiols [reduced glutathione (GSH), cysteine, cysteamine, homocysteine, N-acetylcysteine and coenzyme A]. Furthermore, this method was applied to the selective determination of thiols, mainly GSH and cysteine, in biological samples (rat, liver, kidney, spleen, rat plasma and human serum).

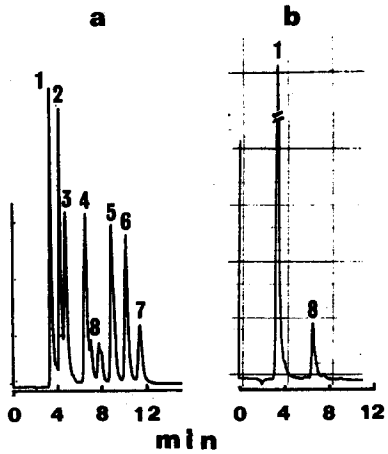

Fig. 2. Chromatograms of DBPM-thiol derivatives (a) and the reagent blank (b). A portion (100 μl) of a standard mixture of thiols (7 nmol/ml each) was treated according to the procedure. Peaks: 1 = internal standard (ANS); 2 = homocysteine; 3 = GSH; 4 = N-acetylcysteine; 5 = cysteine; 6 = cysteamine; 7 = coenzyme A; 8 = peak due to the blank. Peaks at the retention time of ca. 8 min have not been assigned yet.

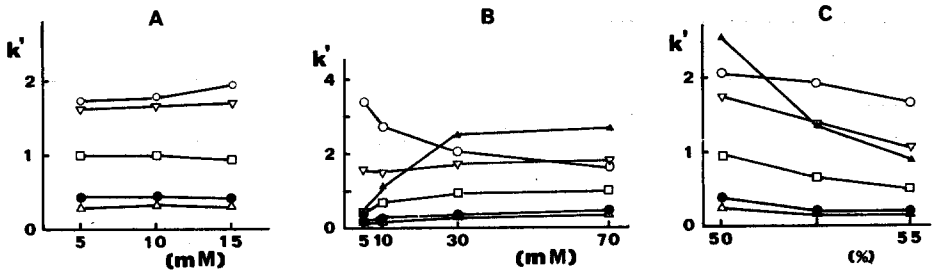

Fig. 3. Effects of (A) phosphate buffer, pH 7.7, (B) tetrabutylammonium bromide, and (C) acetonitrile concentrations on the capacity factor, k'. Data points: ○ = cysteamine; ▽ = cysteine; □ = N-acetylcysteine; ● = GSH; △ = homocysteine; ▲ = coenzyme A.

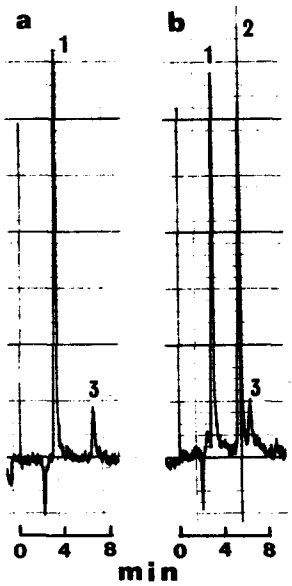

Fig. 4. Separation of the N-acetylcysteine peak from the blank; mobile phase, 10 m*M* phosphate buffer–acetonitrile (105:95, v/v) containing 10 m*M* tetrabutylammonium bromide. (a) Blank; (b) N-acetylcysteine. Peaks: 1 = ANS; 2 = N-acetylcysteine; 3 = unidentified peak.

EXPERIMENTAL

Reagents

Water was deionized and further distilled. Acetonitrile was of HPLC grade (Wako, Osaka, Japan). All other reagents (Wako) were of analytical grade and were not further purified. DBPM was synthesized in our laboratory [13], and purified by thin-layer chromatography on silica gel plates (Kieselgel 60, Merck) using chloroform–benzene (1:1, v/v) as a developing solvent. DBPM for analytical use was recrystallized from acetone and used as an acetonitrile solution. As a diluent, 20 m*M* disodium EDTA was used for the preparation of biological samples.

Preparation of biological samples

Male Wistar rats (seven to eight weeks old) were used. Various organs (liver, kidney and spleen) were removed and perfused with 0.9% (w/v) sodium chloride solution. The tissues were each homogenized in 20 m*M* EDTA solution in a PTFE-glass Potter-Elvehjem homogenizer. The homogenates were individually adjusted to 1% (w/v) (liver and kidney) and 2.5% (w/v) (spleen). Rat plasma was diluted with 20 m*M* EDTA to adjust to 20% (v/v). These homogenates and plasma were used as samples without any deproteinization.

Human serum specimens were obtained from healthy volunteers in our department. Serum was diluted in 20 m*M* EDTA to adjust to 10% (v/v). To 1 ml of this solution were added 0.2 ml of 30% (w/v) metaphosphoric acid. After centrifugation at 2000 *g* at 4 °C for 20 min, 0.5 ml of the supernatant was mixed with 0.24

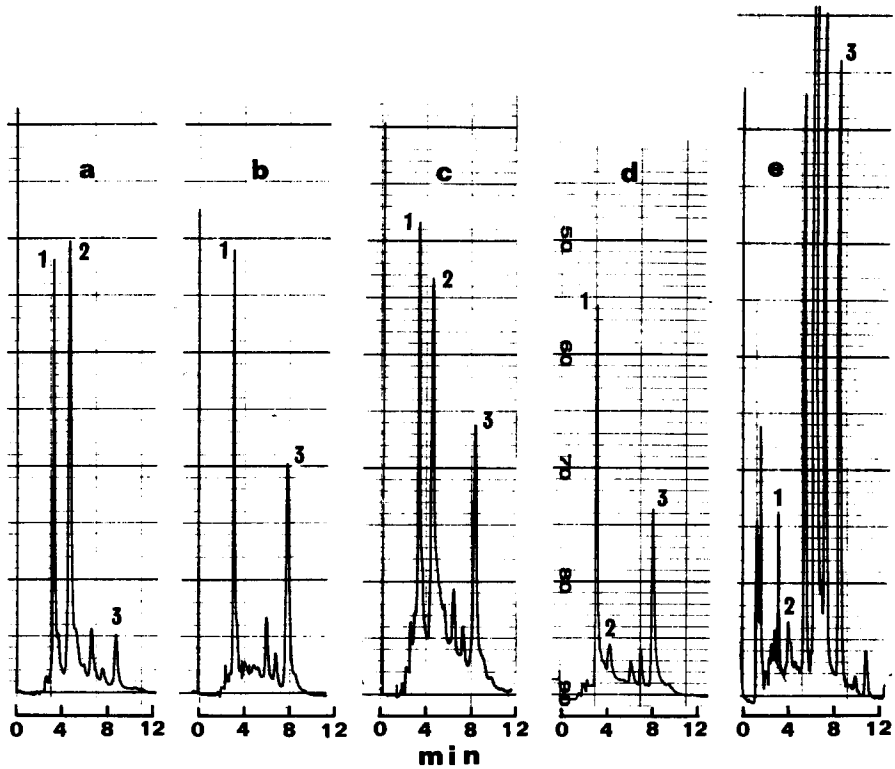

Fig. 5. Chromatograms of thiols following derivatization with DBPM in rat homogenates, rat plasma and normal human serum. A portion (100 μl) of each sample was treated according the procedure. (a) Rat liver; (b) rat kidney; (c) rat spleen; (d) rat plasma; (e) normal human serum. Peaks: 1 = ANS; 2 = GSH; 3 = cysteine.

ml of 2 *M* potassium hydroxide. The resulting solution was used as a sample.

Instrumentation and chromatographic conditions

The liquid chromatograph consisted of a Shimadzu LC-5A pump, a Hitachi Model F1000 fluorescence spectrophotometer operating at an excitation wavelength of 355 nm and an emission wavelength of 457 nm, a Hitachi Model 561 recorder and a Toyo Soda ODS-80^{TM} stainless-steel column (150×4.6 mm I.D., 5 μm).

The eluent for the separation of thiols consisted of a mixture of 10 m*M* phosphate buffer (pH 7.7)–acetonitrile (1:1) containing 30 m*M* tetrabutylammonium bromide. The flow-rate was set at 0.8 ml/min.

Procedure

The incubation mixture consisted of 0.1 ml of a sample, 0.4 ml of 0.1 *M* borate buffer (pH 8.5), 0.3 ml of DBPM–acetonitrile solution (0.24 m*M*) and 0.2 ml of disodium 6-amino-1,3-naphthalene disulphonate (ANS) solution as an internal standard in a vial (5 ml). The mixture was incubated at 60°C for 30 min, then cooled for 5 min in tap water. The mixture was centrifuged at 2000 *g* at 4°C for

TABLE I

CONCENTRATIONS OF GSH AND CYSTEINE IN RAT LIVER, KIDNEY, SPLEEN, PLASMA AND NORMAL HUMAN SERUM

Values indicate mean ± S.D. for the number of determinations shown in parentheses.

Sample		Concentration			
		GSH		Cysteine	
Rat liver	(1%)	6.63 ± 0.56 μmol/g	(4)	0.48 ± 0.06 μmol/g	(4)
Rat kidney	(1%)	N.D.★	(3)	4.24 ± 0.10 μmol/g	(3)
Rat spleen	(2.5%)	0.48 ± 0.06 μmol/g	(4)	1.10 ± 0.19 μmol/g	(4)
Rat plasma	(10%)	4.53 ± 1.01 nmol/ml	(3)	29.0 ± 3.0 nmol/ml	(3)
Human serum	(10%)	6.36 ± 1.55 nmol/ml	(15)	207 ± 35 nmol/ml	(15)

★N.D = non-detectable; trace amount only.

15 min. The supernatant was filtered off through a membrane filter (Millipore, 2 μm) and 20 μl of the filtrate were injected into the chromatograph.

A calibration graph was prepared in the same way, except that the ANS solution was replaced by a solution containing 2.5–100 pmol of thiols. The net peak-height ratios of the individual thiols and ANS were plotted against the concentration of the thiols skipped.

RESULTS AND DISCUSSION

The derivatization reaction conditions were examined on GSH ($2 \cdot 10^{-5}$ *M*). The effect of the DBPM concentration was tested, and constant peak heights were obtained at more than 1.5 times the molar concentration of GSH. Maximum peak heights occurred at 60 °C over ca. 20 min. No pH dependence of the peak height of GSH was observed in the range pH 8–10.

TABLE II

RECOVERY STUDIES OF CYSTEINE AND GSH ADDED TO THE BIOLOGICAL SAMPLES

Sample	Thiol	Amount added (pmol)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)	*n*
Rat plasma	Cysteine	11	101.1 ± 3.96	3.9	3
	GSH	25	106.0 ± 2.36	2.2	3
Rat liver	Cysteine	100	100.2 ± 6.25	6.2	5
	GSH	130	102.4 ± 6.62	6.5	4
Rat kidney	Cysteine	220	103.0 ± 8.36	8.1	4
Rat spleen	Cysteine	450	105.4 ± 5.69	5.4	4
	GSH	420	101.7 ± 3.22	3.2	3

These results agreed well with those from previous studies [14]. The simultaneous separation of DBPM derivatives of six kinds of thiol (homocysteine, GSH, N-acetylcysteine, cysteine, cysteamine and coenzyme A) was examined on the reversed-phase column. Isocratic elution with 10 m*M* phosphate buffer (pH 7.7)–acetonitrile (1:1, v/v) containing 30 m*M* tetrabutylammonium bromide as a mobile phase gave a good separation (Fig. 2a). However, the peak resulting from the reagent blank overlapped with that of N-acetylcysteine, as shown in Fig. 2a and b.

The effect of phosphate buffer, acetonitrile and tetrabutylammonium bromide concentration on the separation is shown in Fig. 3. The peak-height ratios of the individual thiols and ANS were plotted against the thiol concentration. A linear relationship up to at least 1 pmol per 20 μl was obtained. The lower limit of detection at a signal-to-noise ratio of 2 was as follows: homocysteine, 21 fmol; GSH, 17 fmol; N-acetylcysteine, 700 fmol; cysteine, 20 fmol; cysteamine, 50 fmol; coenzyme A, 40 fmol. As described above, the peak due to the reagent blank overlapped that of N-acetylcysteine, and so the detection limit of N-acetylcysteine was not so good. Therefore, the mobile phase was changed for the separation of N-acetylcysteine from the reagent blank by the addition to the mixture of 10 m*M* tetrabutylammonium bromide, when the peak of N-acetylcysteine was well separated from the interfering peak (Fig. 4). Under this condition, the limit of detection of N-acetylcysteine improved remarkably to 20 fmol.

The assay method for thiols in rat tissues, rat plasma and human serum was carried out according to the procedure. Typical chromatograms are shown in Fig. 5. GSH and cysteine were detected predominantly in these biological samples, except for the case of GSH in the kidney (Fig. 5b). Table I shows GSH and cysteine contents obtained by the method. The recoveries of GSH and cysteine added to biological samples are illustrated in Table II.

CONCLUSION

We have developed a sensitive and selective HPLC method for the determination of biological thiols. The method is rapid and can simultaneously separate the six kinds of DBPM derivative of thiols within 12 min. Therefore it can be applied to routine medical studies.

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REFERENCES

1 D. Perrett and S. Rudge, J. Pharm. Biochem. Anal., 3 (1985) 3, and references cited therein.
2 Y. Kanaoka, M. Machida, K. Ando and T. Sekine, Biochim. Biophys. Acta, 207 (1970) 269.
3 Y. Kanaoka, M. Machida, K. Ando and T. Sekine, Anal. Biochem., 48 (1980) 557.
4 Y. Kanaoka, Yakugaku Zasshi, 100 (1980) 973, and references cited therein.

5 J.K. Weltam, R.P. Szaro, A.R. Frackelton, Jr., R.M. Dowben, J.R. Bunting and R.E. Cathou, J. Biol. Chem., 248 (1973) 3137.
6 M. Machida, M.I. Machida, T. Sekine and Y. Kanaoka, Chem. Pharm. Bull., 25 (1977) 1678.
7 M. Machida, T. Takahashi, K. Itoh, T. Sekine and Y. Kanaoka, Chem. Pharm. Bull., 26 (1978) 596.
8 H. Takahashi, Y. Nara and K. Tuzimura, Agric. Biol. Chem., 42 (1978) 769.
9 Y. Nara and K. Tuzimura, Agric. Biol. Chem., 42 (1978) 793.
10 H. Takahashi, Y. Nara, H. Meguro and K. Tuzimura, Agric. Biol. Chem., 43 (1979) 794.
11 O.S. Wolfbeis and H. Marhold. Monatschr. Chem., 114 (1983) 599.
12 K. Nakashima, H. Akimoto, K. Nishida, S. Nakatsuji and S. Akiyama, Talanta, 32 (1985) 167.
13 S. Akiyama, H. Akimoto, S. Nakatsuji and K. Nakashima, Bull. Chem. Soc. Jpn., 58 (1985) 2129.
14 K. Nakashima, K. Nishida, S. Nakatsuji and S. Akiyama, Chem. Pharm. Bull., 34 (1986) 1678.
15 K. Nakashima, K. Nishida, S. Nakatsuji and S. Akiyama, Chem. Pharm. Bull., 34 (1986) 1684.