Journal of Chromatography, 282 (1983) 495–500
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 151

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUOROMETRIC DETECTION OF BIOLOGICALLY IMPORTANT THIOLS, DERIVATIZED WITH AMMONIUM 7-FLUOROBENZO-2-OXA-1,3-DIAZOLE-4-SULPHONATE (SBD-F)

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SUMMARY

Thiol compounds which are derivatized with a fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), are separated on a high-performance liquid chromatography column (μBondapak C_{18}) and detected fluorometrically at 515 nm with excitation at 385 nm. Two kinds of gradient elution system were adopted for the separation of SBD–cysteine, –homocysteine, –cysteamine, –glutathione and –N-acetylcysteine. The detection limits were in the range 0.07–1.4 pmole. Utilizing the proposed method, only a reduced form of glutathione was found in human whole blood at the level of 1.6 ± 0.4 m*M*. However, in the plasma, both the reduced and oxidized (tri-*n*-butylphosphine-treated) L-cysteine, glutathione and an unknown substance were detected. The amounts of L-cysteine and total L-cysteine (reduced plus oxidized) in plasma were 27 ± 2 and 72 ± 7 μM, respectively.

INTRODUCTION

Biological thiols, such as cysteine and glutathione occur widely in living tissues and blood. For the determination of these thiols, various high-performance liquid chromatographic (HPLC) methods, combined with fluorescence detection[1–9] or voltammetric detection[10–17], have been reported. However, the latter methods are not sensitive and selective enough for the measurement of minute amounts of biological thiols. With regard to the fluorometric methods, only a few have been successful in their application to biological specimens.

In a previous paper[18], we reported that a newly synthesized 7-fluoro analogue of SBD-Cl[19], ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), was suitable for determining biological thiols at the picomole level. The high fluorescence and excellent stability of the fluorophores prompted us to use SBD-F for pre-column labelling of thiols in HPLC.

In this paper, we have investigated the HPLC separation of commercially available biological thiols, derivatized with SBD-F and applied the method to blood and plasma samples from normal subjects.

0021-9673/83/$03.00

EXPERIMENTAL

Materials

SBD-F was synthesized according to the previous paper[18]. L-Cysteine (CySH) was obtained from Ajinomoto (Tokyo, Japan). Homocysteine (HomoCySH), cysteamine ($CyNH_2$), glutathione (GSH) and N-acetylcysteine (N-AcCySH) were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade. Methanol for the mobile phase was of HPLC grade (Wako, Tokyo, Japan). Deionized and distilled water was used.

HPLC

A Waters high-performance liquid chromatograph, equipped with a U6K universal injector and a Model 6000A pump were used. For gradient elution, another Model 6000A pump was connected and used under control by a Model 660 solvent programmer (Waters Assoc.). An analytical column of μBondapak C_{18} (300 × 3.9 mm I.D., 8–10 μm), connected to the guard column of Bondapak C_{18}–Corasil (20 × 3.9 mm I.D., 37–50 μm), was used. Phosphate buffer (pH 6.0) and acetate buffer (pH 4.0), used as mobile phases were prepared with 0.1 *M* sodium dihydrogen phosphate and 0.1 *M* disodium hydrogen phosphate, and 0.1 *M* acetic acid and 0.1 *M* sodium acetate, respectively. All buffer solutions used as mobile phases were filtered through a Type HA filter (0.45 μm, Millipore), mixed with methanol and degassed just prior to use. The flow-rate of the eluent was 1.0 ml/min. The column temperature was ambient. The void volume of the column was measured with SBD–cysteine as a marker, eluted with methanol–water (1:1). A Hitachi 650-10S fluorescence spectrophotometer, equipped with an 18-μl flow cell and 150-W xenon lamp, was used with excitation and emission set at 385 ± 10 and 515 ± 10 nm, respectively.

HPLC separation of thiols in whole blood

A 1-ml sample of the fresh blood, drawn with a heparinized syringe, was poured into a disposable tube under cooling with ice–water. To the solution was added an equal volume of a chilled solution of 10% trichloroacetic acid (TCA), containing 1 m*M* EDTA · 2Na. The solution was Vortex-mixed for about 10 sec and then centrifuged at 1850 *g* and 0°C for 5 min. To 20 μl of the supernatant were added 80 μl of water, containing 1 m*M* EDTA · 2Na; 0.2 ml of 2.5 *M* borate buffer (pH 9.5, prepared from 2.5 *M* boric acid and 2.5 *M* potassium hydroxide), containing 4 m*M* EDTA · 2Na; and 0.1 ml of SBD-F [2.0 mg/ml 2.5 *M* borate buffer (pH 9.5)]. Then the solution was vigorously mixed and allowed to stand at 60°C for 60 min. A 10-μl aliquot of the reaction mixture was subjected to HPLC.

HPLC separation of thiols in plasma

The blood was drawn with a heparinized syringe and immediately centrifuged at 1850 *g* and 0°C for 5 min to separate the plasma. To 0.5 ml of plasma was added 0.5 ml of 10% TCA, containing 1 ml of EDTA · 2Na. Then the mixture was immediately Vortex-mixed for about 10 sec and centrifuged at 1850 *g* and 0°C for 5 min. To 0.2-ml portions of the supernatant were added 0.4 ml of 2.5 *M* borate buffer (pH 10.5, prepared from 2.5 *M* boric acid and 2.5 *M* potassium hydroxide), containing 4 m*M* EDTA · 2Na, and 0.2 ml of SBD-F [0.4 mg/ml 2.5 *M* borate buffer

(pH 9.5)]. Then the solution was divided into two equal portions. To one portion was added 10 μl of tri-*n*-butylphosphine (TBP) (0.1 ml/ml) in dimethylformamide (DMF) for reduction of oxidized thiols. Each solution was vigorously mixed and allowed to react at 60°C for 60 min. A 20-μl aliquot of the reaction mixture was subjected to HPLC.

RESULTS

HPLC separation of SBD derivatives

Our first effort was focused on the complete separation of SBD derivatives with a binary eluent on a reversed-phase HPLC column. Methanol concentration and pH of the eluent were investigated by isocratic elution for the separation of SBD-thiols on μBondapak C_{18}. The capacity factors (k') of all the biological thiol derivatives increased as the concentration of methanol decreased (Table I). The acetate buffer (pH 4.0) (isocratic elution IV) gave a smaller k' for the derivatives than did the phosphate buffer (pH 6.0) (isocratic elution II) (Table I). Appropriate resolution was achieved of SBD–cysteine, –glutathione and –N-acetylcysteine by use of each of the two eluents. However, the separation of SBD–homocysteine from –cysteamine was poor. Therefore, gradient elution was investigated. On the basis of the above results, a linear gradient was adopted from solvent III [methanol–0.1 *M* sodium acetate (pH 4.0) (2:98)] to solvent II [methanol–0.1 *M* sodium phosphate (pH 6.0) (5:95)] over 20 min, or from solvent I [methanol–0.1 *M* sodium phosphate (pH 6.0) (1:99)] to solvent VI [methanol–0.05 *M* potassium biphthalate (pH 4.0) (5:95)] over 20 min. As shown in Fig. 1, all the SBD derivatives were well separated in a short time (20 min).

The detection limits (signal-to-noise ratio of 3) for SBD–cysteine, –homocysteine, –cysteamine, –glutathione and –N-acetylcysteine in gradient elution system I

TABLE I

CAPACITY FACTORS (k') OF SBD-THIOLS UNDER VARIOUS ELUTION CONDITIONS

$k' = t - t_0/t_0$; t_0 = 132 sec. Flow-rate, 1.0 ml/min; fluorimeter excitation, 385 nm; emission, 515 nm.

Thiol	*Isocratic elution**						*Gradient elution***	
	I	*II*	*III*	*IV*	*V*	*VI*	*I*	*II*
CySH	2.94	1.77	1.79	1.38	0.63	0.62	1.88	3.23
HomoCySH	4.67	2.68	2.91	2.16	0.94	1.01	3.09	4.89
$CyNH_2$	5.45	3.06	3.57	2.56	0.94	1.01	3.74	5.65
GSH	8.59	3.85	6.07	3.84	2.15	2.64	6.68	6.50
N-AcCySH	11.26	5.20	7.10	4.70	3.03	3.43	8.12	7.15

* Isocratic elution: I = methanol 0.1 *M* sodium phosphate (pH 6.0) (1:99); II = methanol–0.1 *M* sodium phosphate (pH 6.0) (5:95); III = methanol 0.1 *M* sodium acetate (pH 4.0) (2:98); IV = methanol–0.1 *M* sodium acetate (pH 4.0) (5:95); V = methanol–0.1 *M* potassium biphthalate (pH 4.0) (5:95); VI = methanol–0.05 *M* potassium biphthalate (pH 4.0) (5:95).

** Gradient elution: I = methanol 0.1 *M* sodium acetate (pH 4.0) (2:98) to methanol 0.1 *M* sodium phosphate (pH 6.0) (5:95) over 20 min (0 to 100%); II = methanol–0.1 *M* sodium phosphate (pH 6.0) (1:99) to methanol 0.05 *M* potassium biphthalate (pH 4.0) (5:95) over 20 min (0 to 100%).

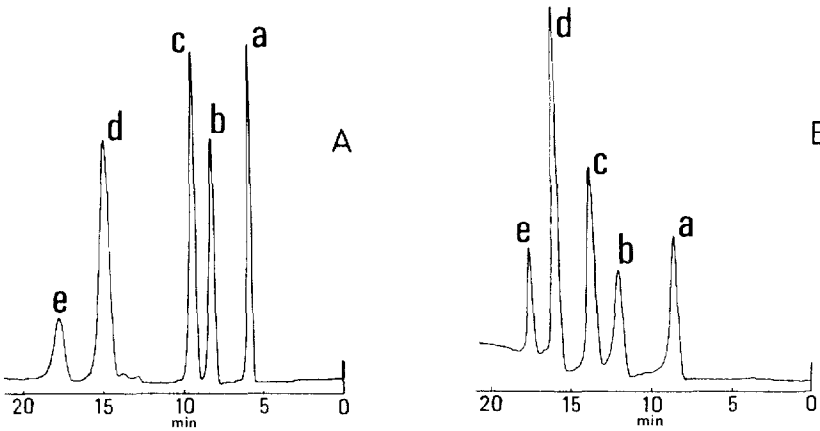

Fig. 1. Chromatograms of biological thiols derivatized with SBD-F. (A) Eluent, methanol–0.1 *M* sodium acetate (pH 4.0) (2:98) to methanol 0.1 *M* sodium phosphate (pH 6.0) (5:95) over 20 min (0 to 100%). (B) Eluent, methanol–0.1 *M* sodium phosphate (pH 6.0) (1:99) to methanol–0.05 *M* potassium biphthalate (pH 4.0) (5:95) over 20 min (0 to 100%). Flow-rate, 1.0 ml/min; fluorimeter excitation, 385 nm; emission, 515 nm. Peaks: a = SBD–cysteine, 30 pmole; b = SBD homocysteine, 7.0 pmole; c = SBD–cysteamine, 3.0 pmole; d = SBD-glutathione, 14 pmole; e = SBD–N-acetylcysteine, 15 pmole. Derivatization procedure: To a 5-ml test tube were added 1 ml of a mixed authentic thiols (CySH 6.34 μM, HomoCySH 1.54 μM, $CyNH_2$ 0.60 μM, GSH 3.03 μM and N-AcCySH 3.18 μM) in 0.1 *M* sodium borate buffer (pH 9.5), containing 2 m*M* EDTA · 2Na and 1 ml of SBD-F (4.85 m*M*) in 0.1 *M* borate buffer (pH 9.5, Na^+). The tube was capped, heated at 60°C for 60 min, and then cooled in ice–water. To the vessel was added 0.1 ml of 2 *M* hydrochloric acid to stop the reaction. A 10-μl sample of the reaction mixture was injected into the column for HPLC.

were 0.60, 0.23, 0.07, 0.45 and 1.1 pmole, respectively (Fig. 1A). In gradient elution system II, the detection limits were 1.4, 0.55, 0.11, 0.30 and 0.62 pmole, respectively (Fig. 1B).

HPLC separation of thiols in whole blood or plasma

As shown in Fig. 2A, only one peak, corresponding to SBD–glutathione, was obtained from whole blood, treated as mentioned in the Experimental section. However, in plasma, two peaks derived from SBD–cysteine and –glutathione appeared with another peak, which is unknown (Fig. 2B). The same trend was observed in plasma treated with TBP added for the reduction of oxidized thiols (Fig. 2C).

DISCUSSION

Among several fluorogenic reagents for thiols[1,6,7,20,21], maleimide-type reagents are mostly used, including N-(7-dimethylamino-4-methyl-3-coumarinyl) maleimide (DACM)[20] which gives a very highly fluorescent adduct. However, the fluorescent adducts are not stable and give two fluorescent products, due to succinimide ring cleavage in the course of time[20]. Therefore, pre-column derivatization with the maleimide type reagents, followed by HPLC, is unfavourable. On the other hand, SBD adducts, which are stable for more than one week at pH 9.5 in a refrigerator, are favourable for that purpose[18].

As shown in the results, the two gradient elution systems are found to be

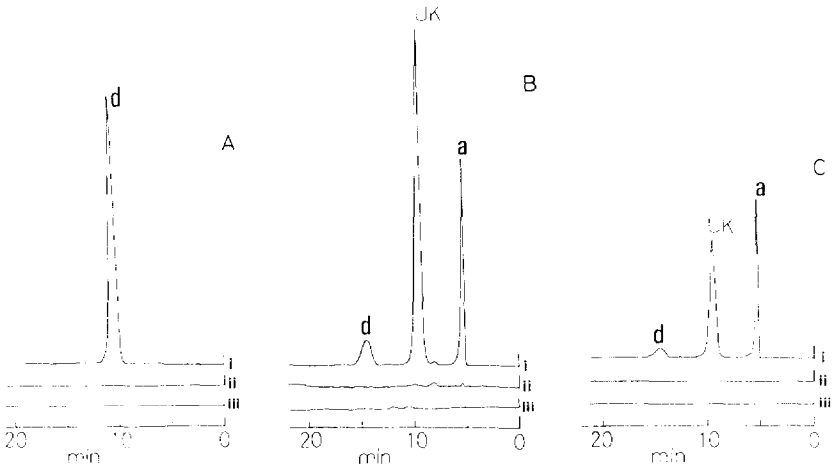

Fig. 2. Chromatograms obtained from whole blood and plasma, derivatized with SBD-F. (A) Isocratic elution; methanol 0.1 *M* sodium phosphate (pH 6.0) (5:95); i = whole blood sample; ii = whole blood without SBD-F; iii = reaction mixture without whole blood. (B) Gradient elution; methanol 0.1 *M* sodium acetate (pH 4.0) (2:98) to methanol 0.1 *M* sodium phosphate (pH 6.0) (5:95) over 20 min (0 to 100%); i = plasma sample; ii = plasma without SBD-F; iii = reaction mixture without plasma. Peaks: a = SBD cysteine; d = SBD glutathione; UK = unknown. (C) Gradient elution; the same as B; i = plasma sample treated with TBP; ii = plasma sample treated with TBP without SBD-F; iii = reaction mixture treated with TBP without plasma. Peaks: a = SBD cysteine; d = SBD glutathione; UK = unknown. Flow-rate of A, B or C, 1.0 ml/min; excitation, 385 nm; emission, 515 nm.

suitable for the separation of biological thiols. Gradient elution system I (III to II over 20 min) was effective for the determination of CySH, HomoCySH and $CyNH_2$. For the determination of GSH and N-AcCySH, gradient elution system II (I to VI over 20 min) might be better, because the peak heights for the latter two SBD-thiols in gradient elution system II are higher than those in gradient elution system I.

With regard to sensitivity, the detection limits for thiols by the present method are almost the same as those by DACM (*ca.* 50 fmole)[22], bimane (*ca.* 1 pmole)[6] or N-(9-acridinyl)maleimide (*ca.* 2.5 pmole)[3], and lower than those by dansylazilidine (*ca.* 15 pmole)[1].

When the proposed method was applied to whole blood or plasma samples, only a few peaks appeared in the chromatograms. According to the previous paper[23], the thiol compounds in blood mainly consist of GSH in its reduced form (1.1 m*M*), mostly concentrated in erythrocytes. The present experiments also confirmed that only a reduced form of GSH is present (more than 90%) at 1.6 ± 0.4 m*M* (male; age, 21–31, $n = 5$). On the other hand, the total amount of cysteine plus cystine in plasma is reported to be 60 ± 7 μM (ref. 24). However, to the best of our knowledge, no reports have appeared on the individual determination of cysteine and cystine in human plasma. According to the present experiments, the amount of cysteine in plasma (male; age, 21–31, $n = 5$) was 27 ± 2 μM and that of total (reduced plus oxidized) cysteine was 72 ± 7 μM.

The unknown peak which appears near the peak of $CyNH_2$ in gradient elution system I separates from the latter peak in gradient elution system II. The compound

corresponding to the unknown peak, which seems to be a thiol of low molecular weight, judging from its small k', is currently being isolated for structure elucidation.

ACKNOWLEDGEMENT

The authors express their thanks to Professor Z. Tamura, University of Tokyo, for his interest and support.

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