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Study of factors affecting the determination of total plasma 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD)–thiol derivatives by liquid chromatography

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Abstract

A detailed investigation of the factors affecting the determination of total plasma 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD)–thiol derivatives (i.e. cysteine, homocysteine and cysteinylglycine) is described. Essentially, this assay entails extracting specific thiols by plasma disulphide bond reduction, protein precipitation, sulphydryl compound derivatization with the thiol-specific fluorogenic reagent ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), and subsequent separation with isocratic reversed-phase high-performance liquid chromatography. By improving the reliability of several analytical parameters (composition of the mobile phase, pretreatment of the sample using different reducing and protein precipitation agents, and optimization of the derivatization of thiols with SBD-F), a number of critical issues can be identified and solved. © 1998 Elsevier Science B.V.

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1. Introduction

The identification and measurement of biological thiol-containing amino acids, such as homocysteine (HCy), cysteine (Cys) and cysteinylglycine (Cys–Gly), have been used as indices of oxidative stress and/or human disease [1–3].

A great amount of evidence suggests that HCy, in particular, may accelerate atherogenesis through vascular endothelial injury [4–6], oxidative modification of low-density lipoproteins [7,8] and/or enhanced lipoprotein a [Lp(a)] binding to fibrin [9], even at physiological concentrations.

Homocysteine is the demethylated derivative of methionine and adenosyl-methionine is the immediate precursor of homocysteine. Following a methyl transfer reaction, adenosyl-homocysteine is hydrolyzed to homocysteine and adenosine. Homocysteine may either be catabolized in the trans-sulfuration pathway via cystathionine to cysteine or remethylated back to methionine [10]. Cysteine is a precursor of glutathione. The tripeptide glutathione (γ-glutamyl-L-cysteinylglycine) and its break-down product, cysteinylglycine [11], maintains the sul-

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phydryl groups of proteins and other compounds in a reduced form.

Since all of these thiols are metabolically related, accurate measurement of them in human plasma is essential.

At present, there is no universally accepted method for determining plasma thiols. Electrochemical detection [12,13], or pre- or post-column derivatization with ninhydrin [14], monobromobimane [15,16], halogenated sulfonylbenzofurazans [17,18], *o*-phthalaldehyde [19,20], followed by high-performance liquid chromatography (HPLC), has become increasingly popular.

The most widely used method for determining total plasma amino thiols (i.e., the sum of free, non-protein-bound, and reduced forms) is based on an existing procedure optimized by Toyo'oka et al. [21], who reported a pre-column derivatization with 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F), after thiol-specific extraction.

The aim of this study is to investigate the factors affecting the SBD-F procedure in detail.

Particular emphasis was placed on (i) the influence of the reducing and deproteinizing agent on thiol plasma recovery; (ii) the use of *N*-acetylcysteine as an appropriate internal recovery standard; (iii) the setting of suitable time intervals and temperatures during the derivatization procedure with SBD-F; (iv) the maintenance of chromatographic resolution and reproducibility with routine and extended use, and enhancement of assay sensitivity.

This improved procedure provides a quantitative and sensitive method that can be adapted to routine measurements of total plasma thiols.

2. Experimental

2.1. Chemicals

L-Cysteine, cysteinylglycine and *N*-acetyl-L-cysteine (NAC), dithioerythritol (DTT), tri-*n*-butylphosphine (TBP) and sodium borohydride ($NaBH_4$) were obtained from Sigma (St. Louis, MO, USA). DL-Homocysteine and SBD-F were obtained from Fluka (Buchs, Switzerland). Acetonitrile and water were of HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of analytical-reagent grade (Aldrich, Milwaukee, WI, USA).

2.2. Chromatographic system

The chromatographic system used was a Waters Model 510A pump equipped with a Rheodyne Model 7125 S injection valve fitted with a 10-μl sample loop. The fluorimetric detection system utilized was a Jasco 820-FP system (Jasco, Tokyo, Japan); the optimal response of SBD derivatives was observed when the excitation and emission wavelengths were set at 395 and 515 nm, respectively.

Separations were performed on a Grom-Sil ODS 2 FE analytical column (125×4.6 mm I.D.; particle size, 3 μm), obtained from Grom Analytik (Herrerberg, Germany). The mobile phase, consisting of 50 m*M* potassium dihydrogen orthophosphate and 1.5% acetonitrile, was adjusted to pH 3.5±0.05 with a 2 *M* solution of orthophosphoric acid. Samples were eluted isocratically at room temperature and at a flow-rate of 1.0 ml/min. The resulting profiles (fluorescence intensity vs. time) were compared with the height of the peaks of the standard solution treated in the same way as patients' specimens.

Stock solutions of Cys (8.2 mmol/l), HCy (7.3 mmol/l), Cys–Gly (5.6 mmol/l),) and NAC (6.1 mmol/l) were prepared in filtered 0.12 *M* perchloric acid and stored at −20°C. Aqueous working solutions (165 μmol/l for Cys, 14.7 μmol/l for HCy, 28 μmol/l for Cys–Gly) were prepared daily.

2.3. Sample collection and preparation

After overnight fasting, blood samples were collected in glass tubes containing EDTA, gently mixed and kept on ice. Plasma was separated by centrifugation within 1 h and stored at −80°C until analysis. No more than three weeks elapsed between sample storage and analysis.

For measurement of total thiol, NAC (as the internal standard; 612 μmol/l) and DTT (500 μmol/l) were added to a 200-μl plasma specimen; the same procedure was performed on an aqueous standard solution. After incubation for 30 min at 4°C, proteins were removed by precipitation with 10% trichloroacetic acid (0.3:1.0, v/v) and centrifugation. The supernatant (50 μl) was then derivatized by adding 100 μl of 0.1 *M* aqueous sodium borate buffer (pH 9.5), 20 μl of 1.5 *M* NaOH and 20 μl of the fluorogenic reagent SBD-F (1 mg/ml dissolved in the above-mentioned borate buffer solution). This

final reaction mixture was heated at 60°C for 10 min, acidified with 10 μl of 0.4 *M* H_3PO_4, cooled on ice and subjected to HPLC analysis with fluorescence detection at room temperature.

2.4. Selection of subjects

The reference group (age range, 20–50 years; median, 33.2 years; males, seventeen; females, nineteen) consisted of 36 healthy hospital staff. All subjects presented values within the normal range of biochemical parameters, together with normal plasma folate, cobalamin and Lp(a) levels. No subjects received oral drugs or multivitamins containing folic acid.

3. Results and discussion

3.1. Optimization of the chromatographic separation

The use of NAC as an internal standard in the determination of plasma thiol was introduced only recently [22,23]. This substance allows more accurate determination than other sulfur compounds, such as mercaptopropionyl-glycine or dithiodiglycolic acid, which are described in literature [24,22]. Although NAC has several disadvantages, such as a long retention time and a consequent wide peak, which affects quantitation, good recovery (99.2±3.0%, range 93–105%) makes it suitable for use as an internal standard.

The chromatographic conditions, reported in Section 2, were specifically designed to allow the best elution of amino thiols and of NAC in particular.

Fig. 1 shows a separation of plasma (B) and of a standard solution (A) obtained within 15 min, when the internal standard peak was still sharp enough to obtain good sensitivity and did not coelute with interference peaks.

Many factors (e.g. mobile phase pH, organic phase concentration, column length, injection volume) strongly affected thiol resolution.

The pH of the mobile phase was a very critical parameter. Fig. 2 shows the influence of pH on the retention time (RT) of amino thiols. A pH range of 2.5–3.0 leads to long RTs, particularly for NAC, which has a RT of over 20 min.

In the pH range 4.0–5.5, NAC elutes quicker. However, Cys–Gly can also be coeluted with HCy.

Optimal results were obtained in the very narrow

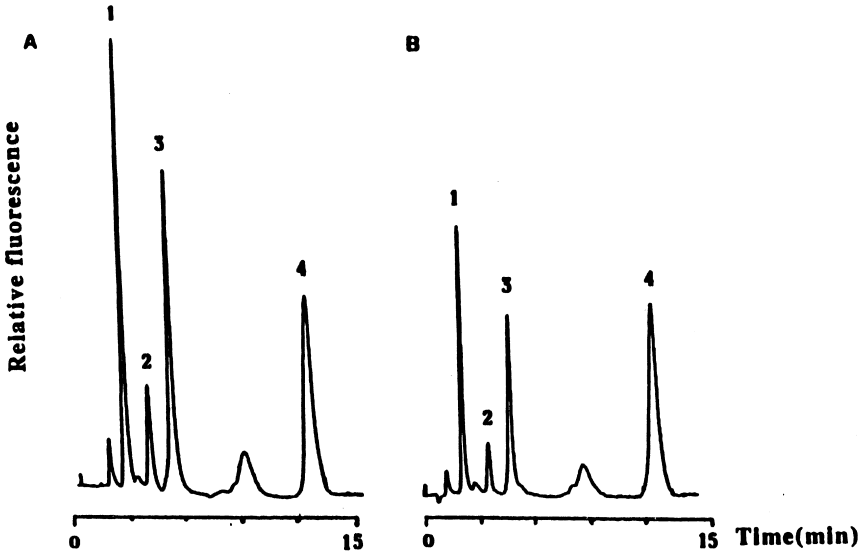

Fig. 1. Typical chromatogram of (A) a SBD-derivatized standard aqueous solution and (B) a SBD-derivatized plasma sample. The standard mixture contained 165 μmol/l of Cys (RT=2′69″), 14.7 μmol/l of HCy (RT=3′70″), 28 μmol/l of Cys–Gly (RT=4′29″) and 612 μmol/l of NAC (RT=13′92″). Plasma sample contained the following concentrations: 107.3 μmol/l of Cys, 6.90 μmol/l of HCy and 16.90 μmol/l of Cys–Gly. Peaks: (1) Cys; (2) HCy; (3) Cys–Gly and (4) NAC.

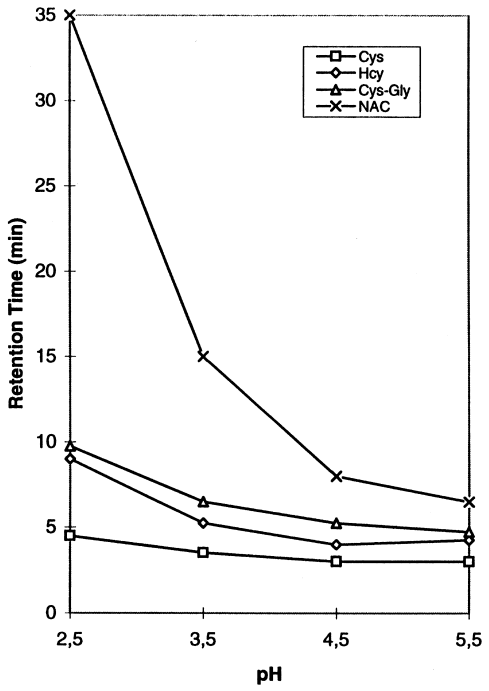

Fig. 2. Influence of pH on the retention time (min) of Cys, HCy, Cys–Gly and NAC.

pH range of 3.5–3.65. This condition allowed good resolution and easy identification of the compound of interest, even in complex matrices such as plasma and urine.

Acetonitrile also strongly affects the elution of the internal standard but has very weak effects on the other components. The acetonitrile concentration must be between 1–3%, because higher concentrations cause fast elution of NAC, which could be coeluted with Cys.

A short column (125×4.6 mm) was chosen in order to obtain lower RTs. Furthermore, a complete column wash requires about 50 min, whereas a longer column (250×4.6 mm) strongly reduces the throughput in routine applications.

High injection volume causes severe broadening of the first-eluting peak (Cys) and decreases the overall chromatographic resolution. To obtain the best separation of the peaks of interest, the optimal injection volume should not be more than 10–15 μl.

3.2. Sample pretreatment

In addition to the analytical methods adopted, sample processing procedures are also a critical factor in thiol analysis. Reduction of the disulfide bonds between HCy and other thiols or proteins is one of the most important steps affecting the final result, since most reducing agents form adducts with fluorogenic reagents, thus influencing the fluorescence intensity of derivatives. For this reason, different reducing agents, such as DTT, TBP and $NaBH_4$ were evaluated. In Table 1, the peak height (fluorescence intensity) of thiol derivatives, obtained from a standard aqueous solution in the presence of different reductants, is compared with the peak height obtained from the same solution without reducing agents. We observed substantial variations in peak height reduction when a reductant-free solution was used as the reference value.

In particular, a 10% solution of TBP (0.01 g/ml of *N*,*N*-dimethylformamide; 2.5 mmol/l final concen-

Table 1
Comparison of the relative fluorescence of the SBD derivatives Cys, HCy, Cys–Gly and NAC using different reducing agents

Reducing agents	Cys		HCy		Cys–Gly		NAC	
	Peak height (cm)	Drop (%)	Peak height (cm)	Drop (%)	Peak height (cm)	Drop (%)	Peak height (cm)	Drop (%)
SBD derivatives without reducing agents	20.1		4.0		10.0		7.2	
SBD derivatives after reduction with TBP (2.5 mmol/l)	9.5	52.7	3.1	22.5	6.0	40.0	4.7	34.7
SBD derivatives after reduction with $NaBH_4$ (1.5 mol/l)	7.7	61.7	2.2	45.0	4.6	54.0	4.6	36.1
SBD derivatives after reduction with DTT (0.5 mmol/l)	6.6	67.2	1.5	62.5	2.0	80.0	2.1	70.8

The fluorescence intensity was obtained using a working aqueous standard solution and was measured as the value of the peak height (cm). Drop (%) is the reduction in peak height (as a percentage), using the values in the absence of reductants as a reference.
The final concentrations of the reducing agents are given in parentheses.

tration) is a useful reducing agent for detecting HCy (peak decrease is only 22.5%), Cys and Cys–Gly. In spite of this good performance in terms of its reaction with SBD-F, TBP is not advisable for routine analysis, due to *N,N*-dimethylformamide toxicity.

$NaBH_4$ (1.5 mol/l final concentration) produces high fluorescence for all thiols examined, but the presence of a fluorescent impurity near HCy makes quantitation difficult. Moreover, it develops gas and foam, leading to inaccurate injection.

The use of DTT as a reducing agent gives the greatest peak decrease. Notwithstanding this, pre-column derivatization with SBD-F after DTT reduction has become increasingly popular. In effect, derivatization in the presence of DTT provides enough sensitivity to allow the sulphydryl compounds to be measured at picomolar concentrations. Moreover, DTT reacts rapidly and specifically with all thiols and forms stable derivatives that are suitable for automation.

To totally reduce the thiols to a single form, a plasma pool was treated, as described above, with increasing amounts of DTT (Fig. 3). The fluorescent response increased with increasing concentrations of the reducing agent, up to 250 μmol/l (final concentration), where a plateau was reached. A concentration of 500 μmol/l was preferred as interfering peaks were absent, even though the responses for Cys and Cys–Gly were lower.

The kinetics of DTT reduction were also studied at different times (from 5 min to 1 h) and at different incubation temperatures (4°C, room temperature and 60°C). Maximal reduction occurs at 4°C and requires up to 30 min.

To remove plasma proteins, several deproteinizing agents [25] were tested. The use of 10% trichloroacetic acid improves assay sensitivity and precision. Orthophosphoric acid, perchloric acid, sulfosalicylic acid, ethanol and acetonitrile were not suitable for thiol detection at very low concentrations. Moreover, the presence of a large peak that eluted with the solvent front could interfere with the determination of Cys.

3.3. Derivatization with SBD-F

SBD-F is a halogenosulfonylbenzofurazan that is used to detect sulphydryl compounds. To obtain the maximum sensitivity of SBD adducts, the fluorescence response was evaluated at different temperatures and reaction times, by testing it with an aqueous standard solution. This fluorogenic reagent reacts quantitatively with thiols at pH 9.5, at 60°C for 10 min, or at room temperature for 1 h.

3.4. Stability

Several studies [26,27] have reported that the concentration of free and total thiols in plasma increases over time if blood is stored after sampling without being centrifuged. We concur with strict

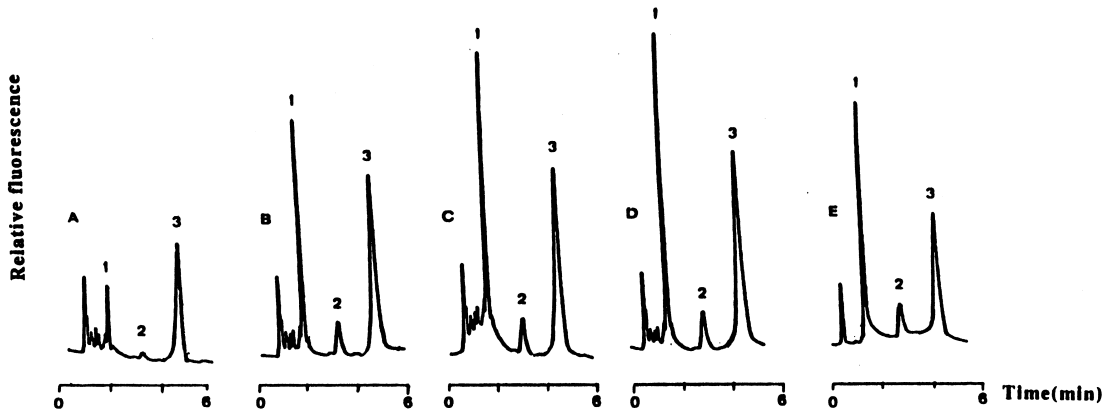

Fig. 3. Effect of increasing amounts of DTT on the relative fluorescence of Cys, HCy and Cys–Gly. Plasma samples were incubated at 4°C for 30 min with (A) 10; (B) 50; (C) 125; (D) 250 and (E) 500 μmol/l DTT. Peaks: (1) Cys; (2) HCy and (3) Cys–Gly.

sampling conditions, but in daily routine clinical analysis, these conditions are difficult to apply. To evaluate the effect of this problem, three aliquots of a blood pool, kept under different storage conditions, were tested. The thiol content in the aliquot that was centrifuged at 4°C immediately after the blood was drawn is similar (<10%) to that found in blood that was incubated at room temperature for 4 and 6 h before centrifugation. These compounds are stable in plasma at room temperature for 24 h, and at 4°C for one week. A nearly unchanged response was observed in the aliquots of plasma kept at 20°C for one month.

SBD derivatives are stable at room temperature for up to 24 h, while, if acidified, they are stable at room temperature for three days. If properly kept at −20°C, they remain unaltered for one month.

3.5. Assay performance

The calibration curve (fluorescence intensity vs. thiol concentration) for Cys, HCy and Cys–Gly was linear in the range 0.01–300 μmol/l. The absolute detection limit, determined as three times the baseline noise level, was 77.6 pmol for Cys, 16.8 pmol for HCy and 13.3 pmol for Cys–Gly. The performance of the assay system was monitored by including control samples. These samples were spiked with known concentrations of thiol compounds and were added with the internal standard, NAC. Blank runs (containing no thiol compounds) gave no peaks under the same chromatographic conditions.

Within-assay precision was calculated by performing ten different control sample extractions on the same day. The coefficients of variation [C.V.(%)] were 0.27% for Cys (mean, 180 μmol/l), 0.98% for HCy (mean, 11.9 μmol/l) and 0.41% for Cys–Gly (mean, 33.6 μmol/l). Between-assay precision was calculated using the results from ten separate determinations obtained within a one-month period; the C.Vs. were 6.25% (mean, 205 μmol/l), 3.2% (mean, 12.5 μmol/l) and 8% (mean, 31.0 μmol/l) for Cys, HCy and Cys–Gly, respectively. The overall recovery was measured using aqueous standard solutions spiked at concentrations ranging from 82.5 to 165.0 μmol/l for Cys, from 14.7 to 73.5 μmol/l for HCy and from 28 to 84 μmol/l for Cys–Gly. The values found, corresponding to Cys=88±4% (range 83–95%), HCy=92±6% (range 80–104%) and Cys–Gly=101±3% (range 95–107%), are in good agreement with the concentrations foreseen, thus demonstrating that the aqueous calibration sample can be used to determine thiols in plasma.

3.6. Plasma reference values

Total thiols were determined in plasma obtained from 36 subjects (seventeen healthy fasting men and nineteen healthy fasting women), age (mean±SD) 33.2±8.2 years (range, 20–50). All of the donors had serum cobalamin, folate and Lp(a) values within the normal range.

As shown in Table 2, the plasma mean concentrations for Cys, HCy and Cys–Gly were 118.6±31.2 (range, 65.5–172), 7.5±2.8 (range, 2.8–15.7) and 17.8±7.4 (range, 5.0–32.0) μmol/l, respectively.

Linear regression showed that both age and sex are not significantly associated with plasma thiol concentrations. These plasma reference values are in agreement with those presented in the most recent literature [28–31].

Table 2
Reference values for total plasma thiols

Thiols	Females		Males		Total	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
Cysteine	113.8±31.9	50–177.6	123.9±30.3	63.3–184.5	118.6±31.2	65–172
Homocysteine	6.9±2.5	2.4–12.1	8.2±3.0	3.2–15.7	7.5±2.80	2.8–15.7
Cysteinylglycine	15.6±6.0	8–29.2	20.3±8.22	5–31.6	17.8±7.40	15–32
Age	34.0±9.5	20–50	32.3±6.6	21–50	33.2±8.23	20–50

Values are expressed as μmol/l.
Number of females=19, number of males=17; total number of subjects=36.

4. Conclusions

Extensive investigation of the parameters influencing SBD–thiol derivatives has demonstrated the following: (i) During the extraction step, a DTT concentration of 500 μM, at 4°C and for 30 min markedly influences the recovery of plasma thiols; (ii) during the oxidation step, the formation of the SBD derivatives was more rapid at 60°C; furthermore, the derivatives are stable at room temperature for several days; (iii) NAC was the compound of choice for use as an internal standard; (iv) this procedure could be adopted as a valid method for routine analysis, except for reduced glutathione (GSH). The low sensitivity of SBD–GSH makes its detection at pico-molar concentrations or less very difficult.

GSH formed during the reduction step can become reoxidized before or after derivatization, and variable reoxidation is a source of non-reproducible results.

As each step was investigated, a reliable, sensitive and adaptable method evolved that could be used to estimate alterations in plasma redox thiol status by accurately determining total HCy, Cys and Cys–Gly.

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