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Pre-column derivatization high-performance liquid chromatographic method for determination of cysteine, cysteinyl–glycine, homocysteine and glutathione in plasma and cell extracts

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Abstract

A sensitive high-performance liquid chromatographic method for quantification of sulphydryl and disulfide amino acids in human plasma using ultra violet spectrophotometric detection was developed. Precolumn derivatization with 5,5′-dithio-bis-nitrobenzoic acid (DTNB) and an optional pre-derivatization reaction with dithiothreitol allowed both quantitative reduction of disulfides for measurement of total amino acid levels and the measurement of the reduced forms. A dynamic range of 500 nmol/l–750 μmol/l allowed the major analytes of interest to be quantified in plasma without sample dilution. The assay is a sensitive and precise method for the determination of sulphydryl and disulfide amino acids in plasma and cell extracts.
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1. Introduction

Biological compounds containing thiol groups are key reactants of intracellular reductive-oxidative metabolic cycles, substrates for protein synthesis and enzyme cofactors. Analytical methods that quantify sulfhydryl amino acids, sulfhydryl peptides, drugs and glutathione frequently rely on high-performance liquid chromatography (HPLC) of chemical derivatives to obtain specific, sensitive and precise results. Many recent reports describe the use of HPLC with fluorescence detection of derivatives [1–8] or electrochemical detection of the sulphydryl groups [9–12], however these detectors are not as widely available as ultra violet (UV)–visible models. To allow quantification with conventional UV-spectro-

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photometric detector, we characterized and refined a rapid HPLC assay of 5,5′-dithio-bis-nitrobenzoic acid (DTNB or Ellman's reagent) derivatives of cysteine, cysteinylglycine, homocysteine and glutathione in human plasma, cell extracts, [13]. The utility of this method was extended to allow determination of total cysteine, total cysteinylglycine, total homocysteine and total glutathione by reducing the disulphides and mixed disulphides present in tissue extracts with dithiothreitol prior to derivitization and analysis. We describe application of this procedure to the analysis of human plasma in renal disease and we anticipate this method can be adapted to a wide variety of investigations.

2. Experimental

2.1. Reagents and chemicals

All reference standards and reagents including L-cysteine, L-cystine, cysteinyl–glycine, γ-glutamyl–cysteine, glutathione, DL-homocysteine, D-penicillamine, 5,5′-dithio-bis-nitrobenzoic acid and dithiothreitol (DTT) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Instrumentation

Chromatographic analysis was performed with a Shimadzu LC-10A liquid chromatograph, SPD-10A variable wavelength spectrophotometric detector, SCL-10A system controller, SIL-10A automatic sample injector and LC-10AT solvent delivery system. Data was collected digitally with Shimadzu Ezchrom Version 3.2 chromatography software.

2.3. Subjects

Volunteer subjects were enrolled in a protocol approved by the University of Saskatchewan Advisory Committee on Ethics in Human Experimentation. Non-fasting venous blood was collected into separate lithium heparin and EDTA vacuum test tubes and cooled on ice. The lithium heparin tubes were immediately centrifuged. Plasma was stabilized within 5 min of venipuncture by adding one volume of 9% (w/v) sulfosalicylic acid, 0.2 m*M* EDTA [13]. Monocytes were harvested from EDTA-blood by centrifugation over ficoll-hypaque and adherence to 30 mm plastic petri dishes for 30 min at 37°C as previously described [14]. Non-adherent cells were removed by rinsing wit phosphate-buffered saline. Monocytes were extracted with 1 ml of 4.5% (w/v) sulfosalicylic acid. Precipitate was removed from either acidified plasma or monocyte extracts by centrifugation at 10 000 *g* for 5 min and the acid supernatant fluid was stored at −70°C for subsequent analysis.

2.4. Derivatization method

The following fluids were sequentially combined: 0.5 ml 0.5 *M* Tris–HCl buffer, pH 8.9; 130 μl acid supernatant fluid, 20 μl of 400 μmol/l D-penicillamine and 350 μl of 10 m*M* DTNB in 0.5 *M* K_2HPO_4, pH 7.2. After 5 min, the mixture was re-acidified by the addition of 50 μl of 7 *M* H_3PO_4, centrifuged and analyzed by HPLC. The re-acidified derivatives were stable for at least 36 h at room temperature.

The reaction mixture for the analysis of total sulfhydryls consisted of 0.5 ml 0.5 *M* Tris–HC1 buffer, pH 8.9; 150 μl of acid supernatant fluid, 20 μl of 400 μmol/l D-penicillamine and 20 μl of 10 m*M* dithiothreitol. After 5 min, 350 μ1 of 10 m*M* DTNB in 0.5 *M* K_2HPO_4, pH 7.2 was added. After an additional 5 min the reaction mixture was re-acidified by the addition of 50 μl of 7 *M* H_3PO_4, centrifuged and analyzed by HPLC.

2.5. Chromatography method

Chromatography of the sulfhydryl-DTNB derivatives was accomplished using isocratic elution on a Supelco LC-18T column (150×4.6 mm, 3 μm) at 37°C. Mobile phase A consisted of 12% methanol (v/v), 100 m*M* KH_2PO_4, pH 3.8 at a flow rate of 1.2 ml/min. Mobile phase B consisted of 40% methanol (v/v), 100 m*M* KH_2PO_4, pH 3.8 at a flow rate of 1.2 ml/min. Analysis was initiated by the injection of 20 ul of the re-acidified derivitization reaction. Sulphydryl-DTNB derivatives were detected by ultraviolet absorbance at 330 nm [13]. After 10 min of isocratic elution with mobile phase A the eluant was changed to mobile phase B in a linear manner during

1 min using the system controller. Mobile phase B was used for 8 min to elute excess DTNB reagent from the column and then the column was re-equilibrated for 7 min with mobile phase A before injection of the next sample.

3. Results and discussion

Ellman's reagent (or DTNB) has been used in a variety of analytical methods of quantifying sulphydryl compounds since its introduction in 1959 by George Ellman [5,15–17]. Most applications rely on complete reaction of sulphydryl containing compounds with Ellman's reagent to produce chromogens that are mixed disulphides. Komuro et al. demonstrated that biological extracts containing cysteine and glutathione can be quantified by pre-column derivitization with Ellman's reagent and UV–HPLC [13]. We have increased the scope of this analytical approach to show that cysteine, cysteinyl–glycine, homocysteine and glutathione can be measured m biological extracts and that oxidized forms of these compounds can also be detected by pre-treating the samples with dithiothreitol.

3.1. Optimization of derivatization

Acid extracts of biological materials are frequently used to prevent oxidation of sulphydryl compounds yet the optimal solubility and reactivity of DTNB occurs in phosphate buffer at pH 8.0. Consequently the derivatization reaction involves the rapid mixing of acid extracts wit DTNB in a high concentration of buffer at pH 8.0. Sufficient DTNB was added to each reaction (3.5 μmol per reaction) to assure complete reaction of glutathione, cysteine and homocysteine (Fig. 1). Note that cysteine and glutathione produced similar chromatographic peak areas, while homocysteine was less chromogenic when equimolar solutions were tested.

3.2. Optimization of DTT mediated reduction of disulphides

Cystine, oxidized glutathione, homocystine and mixed disulphides were reduced and included in analyses by pre-treatment of acid extracts with DTT.

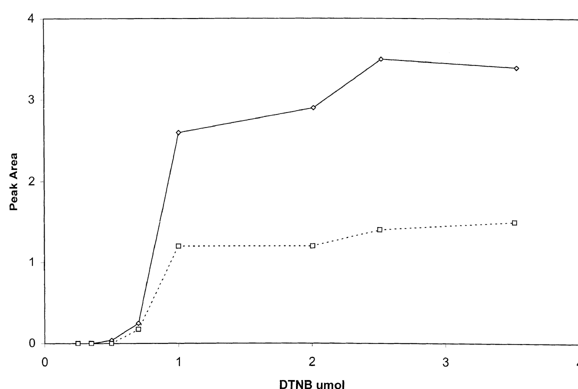

Fig. 1. Influence of DTNB on derivitization of GSH ◊—◊, and homocysteine □---□. DTNB is listed as μmol per reaction. Peak area is in arbitrary units generated by the Ezchrom® software.

Addition of 0.2 μmol of DTT per reaction allowed for complete reduction of disulphides, shown for cystine in Fig. 2. The peak area generated by 450 μmol/l cystine following reaction with 0.2 μmol of DTT was equivalent to the peak area generated by 900 μmol/l cysteine confirming that cystine is quantitatively reduced and derivatized. It should be noted that excess DTT reacts with DTNB leading to higher levels of Ellan's anion in chromatograms and there is sufficient excess of DTNB for complete derivitization following the addition of 0.2 μmol of DTT.

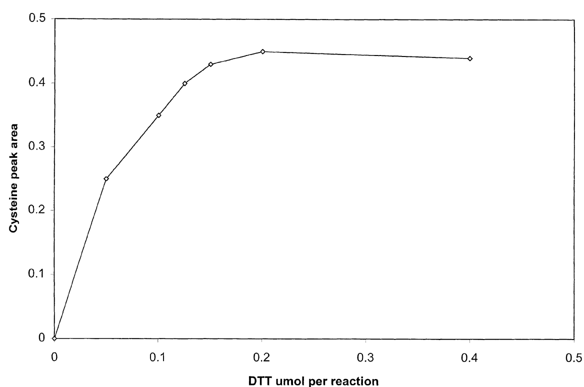

Fig. 2. Influence of DTT on reduction of 450 μmol/l cystine by derivitization and detection as cysteine. DTT is plotted as μmol per reaction. Peak area $\times 10^{-5}$ is in arbitrary units generated by the Ezchrom® software.

3.3. Chromatography

The chromatogram pictured in Fig. 3A confirms there is adequate separation of cysteine, cysteinyl–glycine, Ellman's anion, glutathione and homocysteine from the internal standard (D-penicillamine). The retention time of gamma-glutamyl–cysteine is indicated in Fig. 1A, but it was not quantified. Retention times are listed within Table 1. Analysis of plasma for sulphydryls indicates the presence of cysteine and cysteinyl–glycine and trace amounts of homocysteine and reduced glutathione, Fig. 3B. Following treatment with DTT, the plasma chromatogram peaks contain both the reduced and previously oxidized compounds from disulphides and mixed disulphides, and the peaks for homocysteine and glutathione are more prominent in Fig. 3C. The addition of DTT leads to formation of higher levels of Ellman's anion in the chromatogram Fig. 3C. Derivitization of DTT yields a product that is eluted from the column during the regeneration with mobile phase B and does not interfere with the analysis. This method has also been applied to acid extracts of cells and tissues to produce convenient assays of intracellular glutathione and cysteine. The chromatogram generated for extracts of human monocytes is

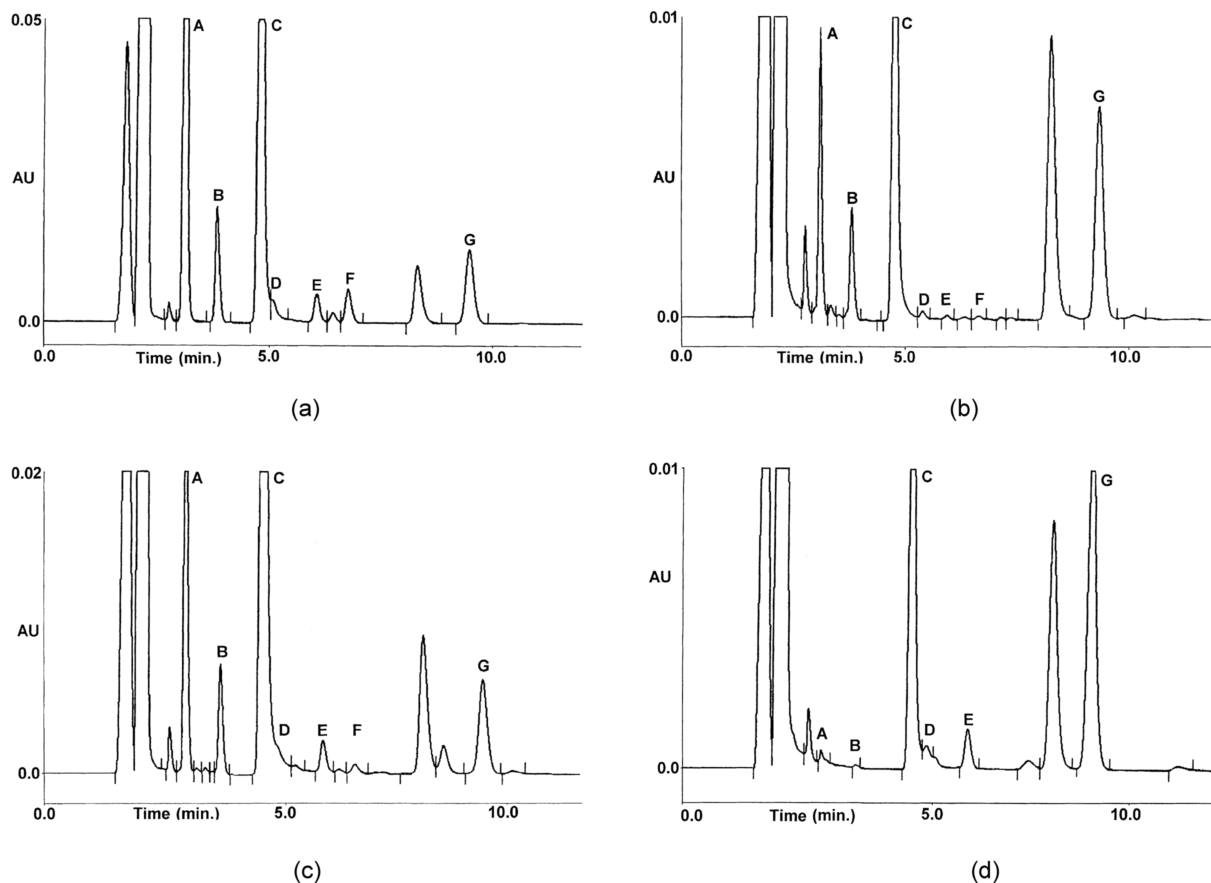

Fig. 3. Chromatograms of (A) aqueous standards of A. Cysteine 750 μmol/l, B. Cys–Gly 80 μmol/l, C. Ellman's anion, D. gamma-glutamyl–cysteine (not quantitated), E. glutathione 30 μmol/l, F. Homocysteine 45 μmol/l, G. Penicillamine (internal standard); (B) Sulphydryl amino acids and glutathione in human plasma; (C) Total sulphydryls and reduced disulphides in human plasma; (D) Sulphydryl amino acids and glutathione in human monocytes.

Table 1
Retention times, analytical range, mid-range coefficients of variation and correlation coefficients (r)[a]

Analyte	Retention time	Analytical range	CV ($n=6$) No DTT	CV ($n=6$) with DTT	r
Cysteine	3.1 min	2–750 μmol/l	2%	2%	0.999
Cys–Gly	3.8 min	0.5–80 μmol/l	4%	5%	0.997
Glutathione	6.1 min	0.5–30 μmol/l	5%	5%	0.999
Homocysteine	6.7 min	0.5–45 μmol/l	6.5%	7%	0.996
Penicillamine	9.7 min				

[a] Correlation coefficients were derived from standard curves using five concentrations.

shown in Fig. 3D indicates that glutathione and cysteine predominate.

3.4. Calibration, precision and analytical range

Routine calibration curves were constructed using two concentrations of each standard for cysteine, cysteinyl–glycine, glutathione and homocysteine as listed in Table 1. Assays were linear within the range of the calibrators, precise and sensitive, Table 1. Recovery for all analytes from plasma ranged from 96 to 98%. The dynamic range of 500 nmol/l to 750 μmol/l allows the measurement of both reduced sulphydryl and total sulphydryl compounds in human plasma at physiological concentrations. Application of these methods to analysis of human plasma in control subjects and subjects with renal disease (pre-dialysis) indicate significant elevations of reduced and total homocysteine and total cysteine in renal disease, while levels of reduced plasma glutathione tend to be lower, Table 2. These results support those recently reported using post-column derivatization methodology [18].

4. Nomenclature

cys–gly	cysteinyl–glycine
DTNB	5,5′ -dithio-bis-nitrobenzoic acid
DTT	dithiothreitol
HPLC	high-performance liquid chromatography

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Table 2
Plasma levels of reduced and total cysteine, cys–gly, glutathione and homocysteine in hemodialysis patients ($n=19$, pre-dialysis) and control patients, ($n=12$), mean±2 standard deviations[a]

Analyte	Control group	Pre-dialysis group	P
Cysteine, μmol/l	9.18±3.02	12.00±7.04	0.14
Cys–Gly, μmol/l	1.62±0.62	1.76±0.93	0.65
Glutathione, μmol/l	0.65±0.35	0.35±0.19	0.02
Homocysteine, μmol/l	0.26±0.25	1.04±0.70	0.0002
Total Cysteine, μmol/l	121.63±31.02	176.15±41.12	0.0003
Total Cys–Gly, μmol/l	12.36±3.23	12.52±5.10	0.92
Total Glutathione, μmol/l	2.71±123	1.95±0.91	0.082
Total Homocysteine, μmol/l	4.32±3.22	7.77±4.29	0.02

[a] Statistical comparison used the unpaired Student's t test.

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