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DETERMINATION OF CYSTEINE, GLUTATHIONE AND N-ACETYLCYSTEINE IN PLASMA BY ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY AND POST-COLUMN DERIVATIZATION

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SUMMARY

The thiol and oxidized forms of cysteine, glutathione and N-acetylcysteine in plasma were determined by ion-pair liquid chromatography and post-column derivatization. The thiol forms were measured after direct injection of deproteinized plasma. The oxidized forms, present either as a dimer or oxidized with other small thiols, were assayed in deproteinized plasma after reduction with dithiothreitol. The total amounts, including the fraction bound to plasma proteins via disulphide bonds, were determined after reductive cleavage in plasma with dithiothreitol. The compounds were separated by ion-pair liquid chromatography on a reversed-phase column (C_{18}) and were detected by fluorimetry after post-column derivatization. The endogenous plasma levels of all forms of cysteine, glutathione and N-acetylcysteine, except for the thiol form of N-acetylcysteine, were above the quantification limits. The quantification limit of N-acetylcysteine as a thiol, was 0.15 μM. The precision was better than 12% for the endogenous concentrations.

INTRODUCTION

Cysteine (CYS) is a precursor of glutathione (GSH) and a metabolite of exogenous N-acetylcysteine (NAC). The low absolute bioavailability of NAC [1,2] may be due to an extensive first-pass metabolism of NAC during which cysteine and other metabolites are formed [3–5].

To be able to study the metabolic pathways and the pharmacological effects of NAC, a method for the simultaneous determination of NAC, CYS and GSH has been developed. In our previous study, a method for measuring NAC, using reversed-phase liquid chromatography (RP-LC) and thiol-selective post-column derivatization with pyrenemaleimide was developed [6]. In the chromatographic system used there, CYS and GSH were eluted within the solvent front. However,

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by using an ion-pair reversed-phase system, CYS and GSH can be retarded and determined simultaneously with NAC.

The objective of the study described here, based on ion-pair RP-LC, was to determine both the thiols and the oxidized forms of CYS, GSH and NAC. This includes oxidation with other thiol-containing compounds of low molecular mass and proteins. As the thiol forms are easily oxidized, careful sample handling and storage are essential for the accuracy of the assay. Deproteinization in plasma was performed immediately after collection and centrifugation of the blood samples. The oxidized forms were measured after reductive cleavage with dithiothreitol, both in plasma and deproteinized plasma.

Previous LC methods for determination of CYS and GLU were based on electrochemical detection [7–9] or pre-column [10–16] or post-column derivatization [17,18].

EXPERIMENTAL

Chemicals and reagents

NAC was purchased from Diamalt (Munich, F.R.G.). GSH (oxidized) and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.), CYS and pyrenemaleimide from Fluka (Buchs, Switzerland). GSH (reduced) and cystine were purchased from Merck (Darmstadt, F.R.G.). Sodium decylsulphate was obtained from Eastman-Kodak (Rochester, NY, U.S.A.), disodium EDTA from Kebo (Stockholm, Sweden) and sodium hydroxide from EKA (Bohus, Sweden). Acetonitrile (HPLC quality), hydrochloric acid (analytical quality), perchloric acid (supra-pure quality) and buffer substances (analytical quality) were obtained from Merck.

Apparatus

The LC system has been described previously [6]. The double Milton-Roy minipump was, however, replaced by two LKB 2150 pumps (LKB, Bromma, Sweden). The pulse dampener of the Eldex pump was modified with a Li-Chrom-A-Damp II (Alltech Assoc., Deerfield, IL, U.S.A.) equipped with a pressure gauge (Alltech Assoc.) connected by a T-coupling.

Chromatographic system

The thiols were separated on a MicroSpher C_{18} (Chrompack, Middelburg, The Netherlands), 3 μm (100 mm $\times$ 4.6 mm I.D.) or a Spherisorb ODS 2, 3 μm (100 mm $\times$ 4.6 mm I.D.) with a mobile phase consisting of 0.6 m*M* decylsulphate and 0.1 m*M* disodium EDTA in 2–6% (v/v) acetonitrile in a phosphate buffer pH 2.0 (μ = 0.01). The flow-rate was 1.0 ml/min. The pH of the mobile phase was adjusted to pH 9.5 with 0.05 *M* borate buffer (pH 11) at 0.5 ml/min before addition of pyrenemaleimide dissolved in acetonitrile at 0.2 ml/min.

Post-column derivatization

The optimal reaction conditions for CYS, GSH and NAC were studied in a flow injection analysis system. PTFE capillaries (0.3 mm I.D.) of different lengths

(1–10 m) were knitted as described by Engelhardt and Neue [19]. Peak areas were measured after injection of 10 μl of the thiol solutions in 0.01 *M* hydrochloric acid. The CYS, NAC and GSH solutions were 0.18, 0.12 and 0.06 m*M*, respectively. The concentration of the pyrenemaleimide solution was 0.05 m*M*.

Blood sample collection

Venous blood samples were collected in chilled EDTA-Vacutainer tubes (Becton Dickinson, Grenoble, France). After immediate centrifugation at +4°C, the plasma proteins in 1.00 ml of plasma were directly precipitated with 0.2 ml of 2.3 *M* perchloric acid containing 7 m*M* dithiothreitol. The supernatant was stored at −70°C. Untreated plasma was stored at −20°C.

Determination of the thiols

For determination of CYS, GSH and NAC, 100 μl of the thawed and mixed perchloric acid supernatant were injected into the column.

Determination of CYS, GSH and NAC oxidized as dimers and with other small thiols

For reduction of disulphide bonds, 125 μl of phosphate buffer (pH 12, μ=2.0), 70 μl of 1 *M* sodium hydroxide and 50 μl of freshly prepared 0.03 *M* dithiothreitol were added to 250 μl of the perchloric acid supernatant, giving a pH of ca. 12. After 20 min at room temperature the mixture was acidified with 15 μl of 11.6 *M* perchloric acid, and 60 μl were injected into the column.

Determination of total CYS, GSH and NAC (including the fractions covalently bound to proteins)

For reduction of disulphide bonds, 375 μl of 0.1 *M* sodium hydroxide and 100 μl of 0.03 *M* dithiothreitol were added to 500 μl of plasma. After 20 min at room temperature the proteins were precipitated with 250 μl of 1.7 *M* perchloric acid. A clear supernatant was obtained by centrifugation, and 50 μl of the supernatant were injected into the column.

RESULTS AND DISCUSSION

Chromatography

RP-LC has been preferred to ion-exchange chromatography for NAC [6] as well as for other thiols [7], because of its higher efficiency and flexibility. Amines, such as CYS and GSH, can be retarded at a low pH as ion-pairs on a reversed-phase column. As the chain length of the counter-ion (hexyl-, octyl-, decyl- and dodecylsulphate, 0.1 m*M*) increased, the retention of CYS and GSH increased and the selectivity decreased. An increase in the concentration of the counter-ion (Fig. 1) produced the expected changes in analyte retentions, e.g. an increase for cationic and a decrease for neutral and anionic compounds (competition for sites). The equilibration time of decylsulphate on the MicroSpher C_{18} column was ca. 4 h (250 ml). The concentration of decylsulphate used, 0.6 m*M*, produced good selectivity and retention times for the thiols in plasma (Fig. 2). After re-

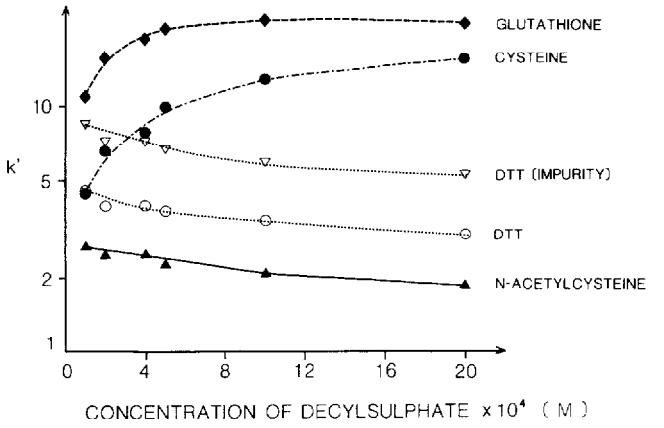

Fig. 1. Influence of the decylsulphate concentration in the mobile phase on the retention of NAC, CYS, GSH, dithiothreitol (DTT) and DTT impurity. Chromatographic conditions: column, MicroSpher C_{18}, 3 μm (100 mm×4.6 mm I.D.); mobile phase, 2% (v/v) acetonitrile in phosphate buffer (pH 2.0) containing 0.1 m*M* EDTA and various concentrations of decylsulphate.

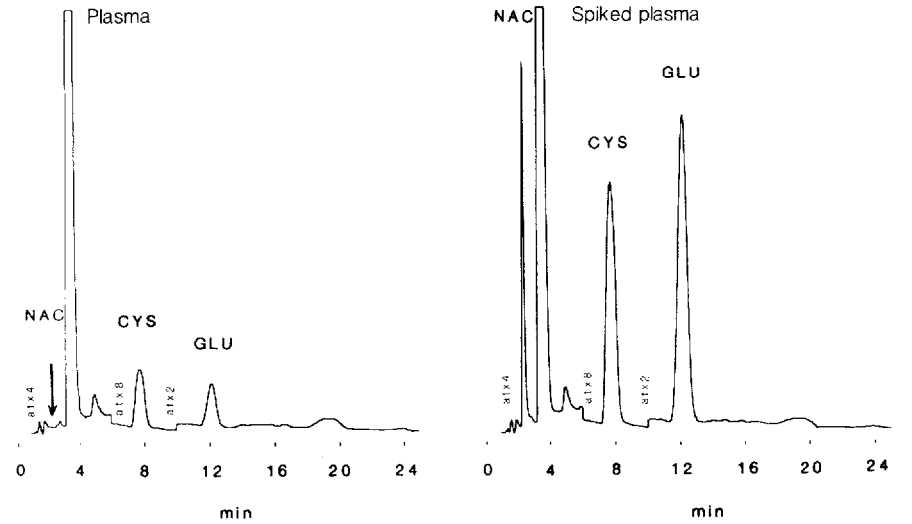

Fig. 2. Blank plasma and spiked plasma chromatograms of the thiol forms of NAC, CYS and GSH. Injection volume, 100 μl. The sample was spiked with 6.4 μ*M* NAC, 23.4 μ*M* CYS and 11.2 μ*M* GSH. Chromatographic conditions: column, Spherisorb ODS 2, 3 μm (100 mm×4.6 mm I.D.); mobile phase, 0.6 m*M* decylsulphate and 0.1 m*M* EDTA in 6% (v/v) acetonitrile in a phosphate buffer (pH 2.0).

duction of the disulphides in deproteinized plasma (Fig. 3) and in plasma (Fig. 4), three late-eluting peaks appeared. The retentions of these endogenous compounds were affected in a similar way to those of the amines, so it was not possible to reduce their retention times in a simple way. Furthermore, GSH and the late-eluting endogenous compounds were not separated on a short C_{18} column (Perkin-Elmer, 30 mm×4.6 mm I.D.), so a heartcut in a coupled column system could

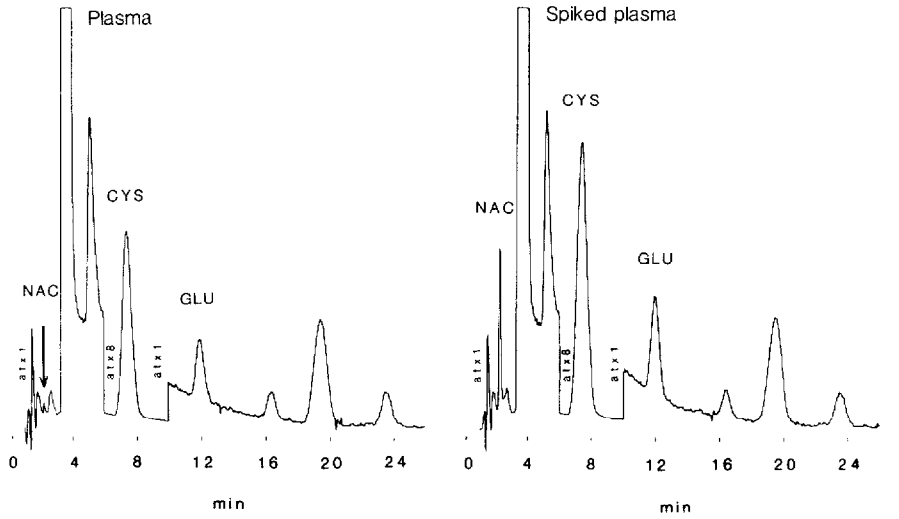

Fig. 3. Blank plasma and spiked plasma chromatograms of NAC, CYS and GSH, after reduction of oxidized forms in the supernatant. The sample was spiked with 2.8 μM NAC, 59.7 μM CYS and 3.9 μM GSH. Injection volume, 60 μl. Chromatographic conditions as in Fig. 2.

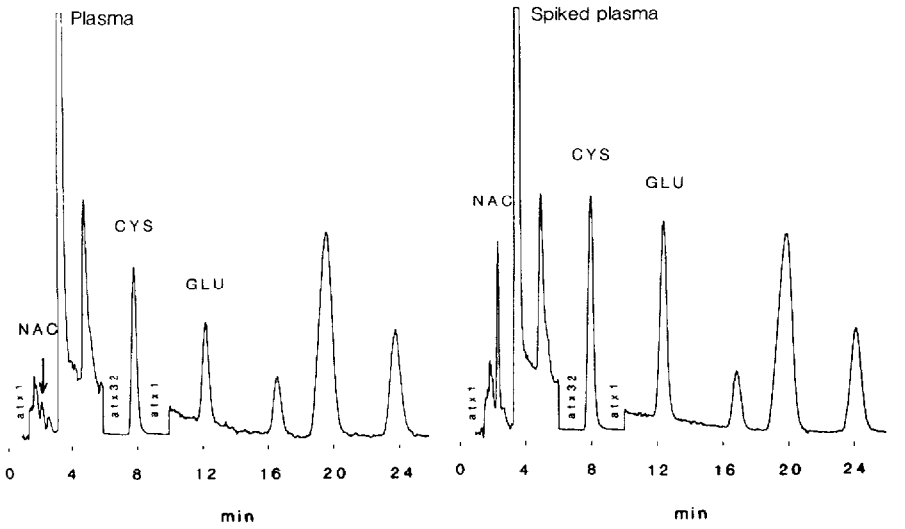

Fig. 4. Plasma blank and sample chromatograms of total concentration of NAC, CYS and GSH. The sample was spiked with 3.4 μM NAC, 114 μM CYS and 7.6 μM GSH. Injection volume, 50 μl. Chromatographic conditions as in Fig. 2.

not be used to reduce the analysis time. The retention of the late-eluting compounds decreased more than that of the early-eluting NAC if the acetonitrile content was increased. The best results regarding selectivity and analysis time were obtained using the chromatographic system given in Fig. 2.

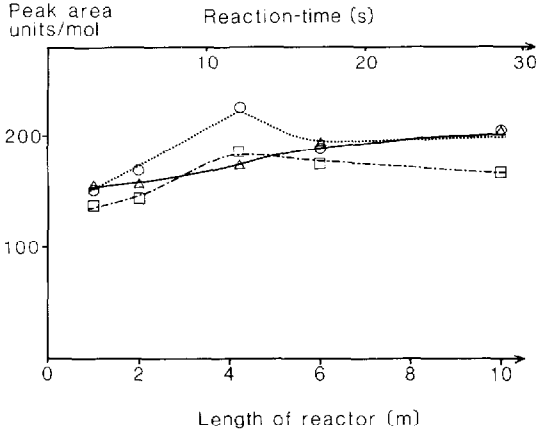

Fig. 5. Influence of the length of the knitted PTFE capillary on the peak area of the fluorescence signal of NAC (○), CYS (□), and GSH (△). Conditions: mobile phase, 2% (v/v) acetonitrile in phosphate buffer (pH 2.0) containing 0.5 m*M* decylsulphate and 0.1 m*M* EDTA; flow-rate, 1.0 ml/min; buffer, 0.05 *M* borate buffer (pH 11.0); flow-rate, 0.5 ml/min; reagent, $5 \cdot 10^{-5}$ *M* pyrenemaleimide; flow-rate, 0.2 ml/min; injection volume, 10 μl of 0.12 m*M* NAC, 0.18 m*M* CYS and 0.06 m*M* GSH; detection, fluorescence 342/389 nm.

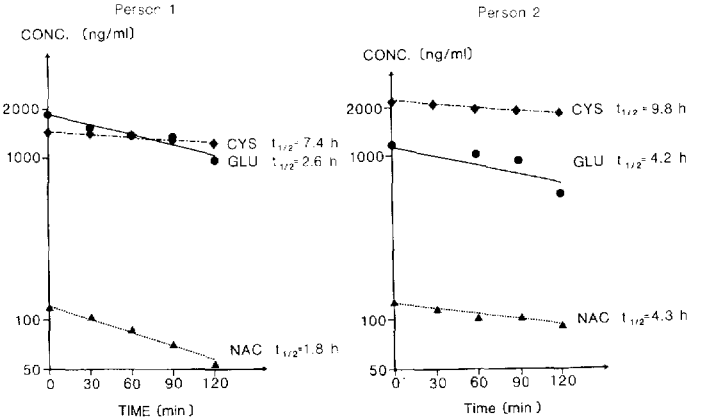

Fig. 6. Half-lives ($t_{\frac{1}{2}}$) of NAC, CYS and GSH in two human whole blood samples during storage in ice-water before centrifugation. The concentrations refer to those determined in the plasma after centrifugation.

Derivatization

Pre-column and post-column derivatization of NAC with pyrenemaleimide have been compared previously [6]. The post-column technique is preferable as the sample preparation is simpler (cf. refs. 6 and 20). The derivatization comprises a nucleophilic addition of the thiol to the carbon–carbon double bond of the reagent. The reaction rate of different compounds may depend on the pK_a value of the thiol, since the thiolate ion is a stronger nucleophile. The optimal reaction times for the post-column derivatization of NAC, CYS and GSH were investigated using a flow injection analysis system. Knitted capillaries (0.3 mm I.D.) of

TABLE I

STANDARD CURVES FOR THE DIFFERENT FORMS OF NAC, CYS AND GSH

Standard curve equation: $y = a + bx$; r^2 = coefficient of determination.

Compound	Concentration range (μM)	r^2	a	b
Thiols				
NAC	0.18- 6.1	0.9995	0.24± 1.7	35.1 ±0.4
CYS	2.5 - 25	0.9997	3.4 ± 8.8	39.8 ±0.3
GSH	0.65- 16	0.9992	2.3± 4.2	27.8 ±0.4
Small disulphides				
NAC	0.3 - 11	0.9993	2.5 ± 1.1	10.6 ±0.1
CYS	50 -248	0.9998	−12.9 ±18.6	12.5 ±0.1
GSH	3.9 - 39	0.9971	−2.3 ± 6.3	8.9 ±0.2
Total				
NAC	0.3 - 18	0.9994	1.6 ± 1.1	8.3 ±0.1
CYS	80 -412	0.9999	5.7 ±10.9	10.41±0.04
GSH	3.3 - 33	0.9904	5.7 ± 5.5	7.1 ±0.3

different lengths were used (Fig. 5). The maximum peak areas of NAC and CYS were reached at 11.3 s. GSH did not reach a maximum level within 29 s. It was impossible to use a longer capillary with 0.3 mm I.D. since the back-pressure became too high. The longer reaction time for GSH could not be explained by differences in pK_a values, since the pK_a values of the thiol functions of NAC, CYS and GSH were 9.6, 10.8 and 9.2, respectively [21,22]. The pH in the capillary was 9.8. A more probable explanation is that there is an intrinsic steric hindrance of the larger glutathione molecule.

Sample handling and sample stability

Careful sample handling, work-up and storage are important if oxidation of the thiols is to be avoided. Non-enzymatic oxidation of GSH is catalysed by oxygen, copper ions and a thiol–disulphide interchange, mainly with cysteinylglycine [23]. The in vitro half-lives in rat plasma have been determined as 4 min for GSH and 2 min for CYS [22]. These values were considerably lower than those of CYS, GSH and NAC in two human whole-blood samples stored in ice-water. NAC was most easily oxidized (Fig. 6) with half-lives of 1.8 and 4.3 h. CYS had half-lives of 9.8 and 7.4 h and the values for GSH were 2.6 and 4.2 h. Oxidation of less than 8, 2 and 5% for NAC, CYS and GSH, respectively, occurred if centrifugation and protein precipitation were performed within 10 min. Only plasma samples without any sign of haemolysis can be analysed, since GSH has a high distribution to blood cells [24]. Dithiothreitol has recently been shown to be more suitable reducing agent for disulphides than either sodium borohydride or tributylphosphine [25]. The reduction of NAC with dithiothreitol is faster at a high pH [6]. No oxidation of NAC, CYS or GSH occurred in the prepared samples within 20

TABLE II

WITHIN-DAY PRECISIONS AND ABSOLUTE RECOVERIES OF THE DIFFERENT FORMS OF NAC, CYS AND GSH

Compound	Added (μM)	Found* (μM)	Absolute recovery* (%)	Relative standard deviation* (%)	n	Injection volume (μl)
Thiols						
NAC	–	–	–	–	10	100
	0.157	0.15	96	5.4	10	100
	6.20	6.15	99	3.1	10	100
CYS	–	9.15	–	1.0	10	100
	14.9	25.7	111	1.6	10	100
	24.8	35.9	108	1.2	10	100
GSH	–	1.70	–	2.7	10	100
	7.81	10.4	111	3.0	10	100
	13.7	15.5	101	1.5	10	100
Small disulphides						
NAC	0	0.41	–	7.8	10	100
	2.87	3.27	100	2.3	9	60
	16.3	16.6	100	1.5	10	60
CYS	0	120	–	0.8	10	60
	59.8	187	112	0.9	10	60
	116	249	111	0.8	10	60
GSH	0	4.43	–	11.1	10	60
	4.01	8.46	100	6.7	10	60
	8.01	12.8	104	5.4	10	60
Total						
NAC	–	0.71	–	6.2	10	100
	3.33	3.99	98	4.0	10	50
	14.8	15.3	99	0.7	10	50
CYS	–	244	–	0.6	10	50
	50.4	299	109	0.6	10	50
	114	372	112	0.7	10	50
GSH	–	6.43	–	7.9	10	50
	9.62	15.8	97	4.7	10	50

*Peak-height measurement.

h, if they were stored in the automatic injector chilled to $+4^{\circ}C$. The thiols in the acidic supernatant containing dithiothreitol were stable at $-70^{\circ}C$ for more than six months.

Quantitative determinations

The concentrations in plasma of NAC, CYS and GSH differ widely, as do the proportions of the different forms (oxidized or reduced) in which they appear. The linear range of the standard curve increased with increasing concentration of the pyrenemaleimide reagent, but so did the baseline noise. The concentration

of pyrenemaleimide used for derivatization of the thiol forms was 0.03 m*M* and for the oxidized forms it was 0.08 m*M*. Precipitation of the reagent occurred in the knitted reactor at a concentration of 0.34 m*M*.

In routine determinations, calibration curves of the thiol forms of NAC, CYS and GSH in phosphate buffer (pH 7.4) were prepared every day in the actual concentration range. Typical standard curves of the different forms of thiols are shown in Table I.

The endogenous concentrations of the different forms of NAC, CYS and GSH are above the quantification limit, except for NAC as a thiol, which has a quantification limit of 0.15 μM. Within-day precision and absolute recoveries of the different forms of NAC, CYS and GSH are shown in Table II. In order to determine the precisions and absolute recoveries of the thiols, and of the total concentrations, the thiol forms were added to plasma. To measure the precisions and absolute recoveries of disulphides, the dimers were added to plasma (NAC-NAC was prepared according to ref. 6). The absolute recoveries were compared by direct injection of NAC, CYS and GSH dissolved in 0.34 *M* perchloric acid containing 0.6 m*M* dithiothreitol.

CONCLUSIONS

Post-column derivatization has proved to be a suitable technique for the measurement of thiols. The reaction with pyrenemalemide is rapid and reproducible for NAC, CYS and GSH. Reversed-phase ion-pair chromatography with decylsulphate produces a good selectivity between NAC, the endogenous thiols and the reducing agent dithiothreitol. This facilitates sample handling for thiols, since dithiothreitol can be added as a stabilizer before the deproteinized acid plasma samples are frozen. Furthermore, as the excess dithiothreitol after reduction of the disulphides does not have to be separated before injection, the work-up procedure becomes very simple and ca. 40–50 samples can analysed every day using an automatic injector.

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