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APPLICATION OF CAPILLARY ISOTACHOPHORESIS TO THE ANALYSIS OF GLUTATHIONE CONJUGATES

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SUMMARY

An analytical capillary isotachophoretic method has been applied for the quantitative assay of reduced glutathione (GSH) conjugates produced by the cytosolic enzyme GSH S-transferase. The donor molecule GSH in reduced and oxidized (GSSG) forms and the GSH conjugates of at least two electrophilic acceptors can be assayed in a single analysis. This technique also permits the quantitative assay of further metabolites of GSH conjugates including mercapturic acids. The total analysis time was of the order of 30 min. The sensitivity of the method is sufficient for the accurate detection of 0.15 nmol GSH conjugate of 1-chloro-2,4-dinitobenzene and *p*-nitrobenzyl chloride, 0.2 nmol GSH conjugate of 1,2-epoxy-3-(*p*-nitrophenoxy)-propane and 0.15 nmol GSH. The present method is simple, accurate and does not require radioactively labelled compounds or separate analytical procedures.

INTRODUCTION

Reduced glutathione (GSH) S-transferases (GST) (E.C. 2.5.1.18) are a key phase II enzyme system, initiating the detoxification of potentially alkylating physiological and exogenous agents including drug metabolites[1–4]. These enzymes catalyze the conjugation of GSH with a variety of electophilic toxic compounds to GSH conjugates. The latter are further metabolized in the mercapturic acid pathway to the corresponding mercapturic acids, which are excreted in bile and urine.

Several analytical methods have been developed for the quantitation of GSH conjugates, including radioimmunoassay[5], ion-exchange high-performance liquid chromatography (HPLC)[6,7] and reversed-phase HPLC (RP-HPLC) with UV and fluorescence detection[8,9]. The ion-exchange HPLC method with precolumn derivatization with *o*-phthaldialdehyde, recently described by our group[7], is suitable for the detection of all GSH conjugates and GSH. However, this method does not allow the determination of mercapturic acids. By using the RP-HPLC method with UV detection, GSH conjugates and mercapturic acids can be sensitively detected but not GSH and oxidized glutathione (GSSG)[8]. Furthermore, the chromatographic methods require particularly large sample volumes and elaborate sample preparation. An optimum method for the investigation of metabolic studies would allow simultaneous measurement of all these major compounds of the mercapturic acid pathway.

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Capillary isotachophoresis has proved useful for the quantitation of a wide range of ionogenic organic compounds and inorganic ions. Recently, this technique has been reported to allow the simultaneous analysis of GSH in reduced and oxidized forms[10]. Since all GSH conjugates involve GSH in their molecules, they could theoretically also be determined by capillary isotachophoresis. Holloway and Battersby[11] have recently reported that this technique can also be applied to the determination of GSH conjugates. Parallel and independent of this group, we investigated the applicability of analytical capillary isotachophoresis to the analysis of GSH conjugates and mercapturic acids. Capillary isotachophresis offers three major advantages: (a) a rapid analysis without extraction steps; (b) detection of GSH conjugates of which the original substrates do not show UV absorbance, fluorescence or radioactivity and (c) simultaneous detection of GSH, GSH conjugates and mercapturic acids.

This paper demonstrates the applicability of the analytical capillary isotachophoretic technique to the analysis of GSH conjugates. This technique has been applied[10] for the study of GSH conjugation reactions catalyzed by cytosolic glutathione S-transferase (GST). Furthermore, it has been extended to the determination of mercapturic acids.

EXPERIMENTAL

Chemicals and reagents

GSH, GSSG, N-acetylcysteine, 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and the GSH conjugate of *p*-nitrobenzyl chloride (PNBC) were obtained from Sigma (München, F.R.G.), phenylglycidether (PGE) from Fluka (Neu-Ulm, F.R.G.), styrene oxide (SE) from Janssen (Nettetal, F.R.G.) and hydroxypropylmethylcellulose (HPMC) from Ega-Chemie (Steinheim, F.R.G.). These chemicals were of the highest quality available. All other chemicals were from Merck (Frankfurt/Main, F.R.G.).

Exogenous conjugates (except for PNBC-SG) and mercapturic acids were prepared non-enzymatically from the corresponding substrates and GSH or N-acetylcysteine in an ethanolic solution of sodium hydroxide at 25°C. Buffer solutions of these substances were used as standards for calibration of zone lengths.

Cytosol containing large amounts of GST was prepared from liver homogenates of rats by a standard centrifugation technique (200 000-*g* fraction) using 0.1 *M* Tris–HCl, pH 7.4, containing 1 m*M* ethylenediaminetetraacetic acid (EDTA) and 1.15 g% KCl for homogenization of the fresh liver (20%, w/v). Protein concentrations were determined by the method of Bradford[12].

Apparatus

The isotachophoretic analyses were performed on the LKB 2127 Tachophor (Bromma, Sweden) fitted with a poly(tetrafluoroethylene) capillary (23 cm × 0.5 mm I.D. and 40 cm × 0.5 mm I.D.). All analyses were carried out at ambient temperature. The zones were detected by UV (254 nm) and conductivity detection.

Methods

Two electrolyte systems were employed. System A: the leading electrolyte system was 5 m*M* HCl, corrected to pH 7.0 by the addition of Tris, and containing 0.25 g%

HPMC. The terminating electrolyte was 10 m*M* phenol, adjusted to pH 10.0 by the addition of freshly prepared and filtered barium hydroxide solution. System B: the leading electrolyte was 10 m*M* HCl, corrected to pH 3.3 by the addition of β-alanine, and containing 0.25 g% HPMC. The terminating electrolyte was 10 m*M* hexanoic acid. System B was previously employed by our group in the assay of glucuronides derived from phenolic compounds[13]. Separations were carried out at an initial constant current of 50 μA, which was reduced to 25 μA when the voltage reached 10 (system A) or 8 kV (system B). The terminator passed the detectors at a potential of around 10 (system A) and 8.4 kV (system B) at 25 μA. The injection volume was 1–5 μl. The total analysis time was in the order of 30 min.

GSH conjugation reactions were carried out in 0.1 *M* Tris–HCl or 0.1 *M* potassium phosphate buffer, each of pH 6.5, containing GSH at a concentration of 2.5–5 m*M*. The acceptor concentration was varied in the range of 0.5–5 m*M*. Because of the low water solubility of the acceptors, their solutions were prepared in ethanol. The final ethanol concentration in the assay mixture was less than 5% (v/v). The total protein concentration in the assay mixture ranged from 0.1 to 1.0 mg/ml. The enzymatic reaction was stopped by rapid mixing with ice-cold methanol (1:1, v/v). As references for the isotachophoretic analysis of the GSH conjugation reactions, photometric and HPLC techniques were employed[1,14].

The width of an UV-absorbing zone was measured at half its height and was converted into units of time according to the set chart speed. The width of a non-UV-absorbing zone was estimated at half the height of the lower absorbing neighbouring peaks, which can either be sample zones or a peak due to an impurity.

RESULTS AND DISCUSSION

Fig. 1 shows isotachopherograms of an incubation mixture at an early and later stage of the enzymatic GSH conjugation of SE, respectively. GSSG and SE-SG appear as slight UV-absorbing zones, while GSH gives a non-UV-absorbing zone. GSSG shown in Fig. 1 is derived from GSH by autooxidation during incubation. Fig. 1 demonstrates that GSH conjugates, the cosubstrate GSH and the non-conjugating GSSG can be determined in a single analysis. Since all electrophilic substrates used in this study are neutral molecules, they cannot be detected by this method. The order of migration of the organic anions involved in this study from high to low mobility was GSSG, GSH, mercapturic acid and GSH conjugate by using both electrolyte systems.

The calibration graphs obtained by injection of the reference substances were linear for the range 1–25 nmol. Fig. 2 shows an example for GSSG, GSH and SE-SG. The slopes of the calibration plots for GSH, GSSG, some GSH conjugates and the mercapturic acid of 1-chloro-2,4-dinitrobenzene (CDNB) by using both electrolyte systems are given in Table I. As can be seen from this Table, the zone widths of all substances measured by using system A were longer than those obtained by using system B. These differences may result from the different pH values of the leading electrolytes used in the two systems.

Fig. 3a–d show isotachopherograms obtained by using system A from separate incubations of CDNB, PNBC, EPNP and PGE with GSH and rat liver cytosolic GST. Fig. 4a and b show isotachopherograms from a separation of a mixture of PNBC-SG and PGE-SG obtained by using systems A and B respectively. Complete separation

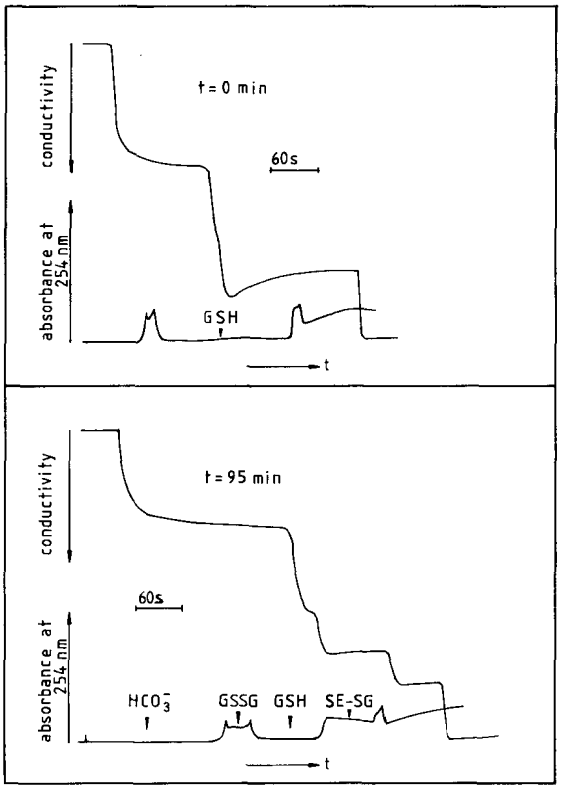

Fig. 1. Isotachopherograms from an incubation of SE (4.4 m*M*) with GSH (5 m*M*) and cytosolic GST (0.3 mg/ml) at $t = 0$ min and 95 min. System A, 23-cm capillary.

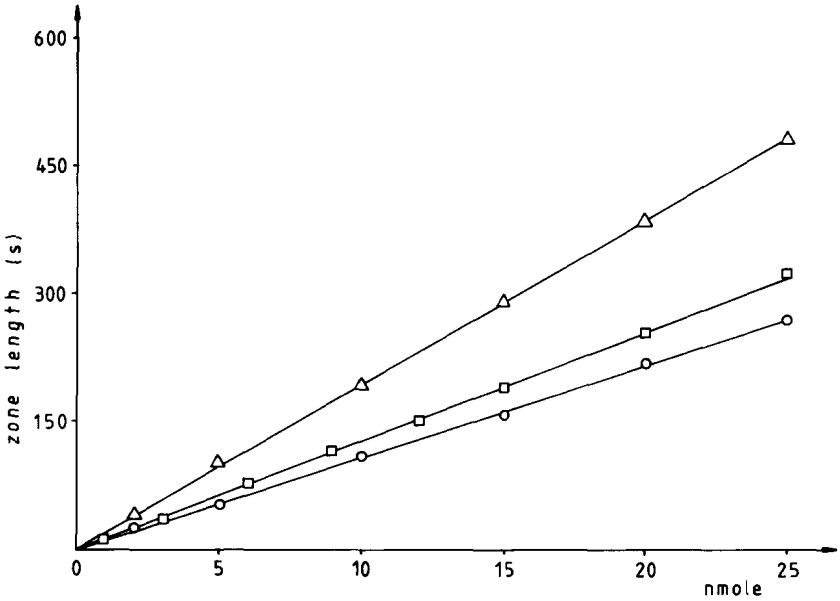

Fig. 2. Calibation graphs for GSH ( ). GSSG (△) and SE-SG (□). System A, 23-cm capillary.

TABLE I

ZONE LENGTHS FOR A SERIES OF SUBSTANCES OBTAINED IN THE TWO ELECTROLYTE SYSTEMS

Substance injected	*Slope of plot (s/nmol)*	
	System A	*System B*
GSH	10.4	7.0
GSSG	18.9	12.1
SE-SG	12.5	–
PGE-SG	13.2	7.7
PNBC-SG	12.8	8.1
EPNP-SG	12.1	7.2
CDNB-SG	11.8	–
CDNB-mercapturic acid	12.0	–

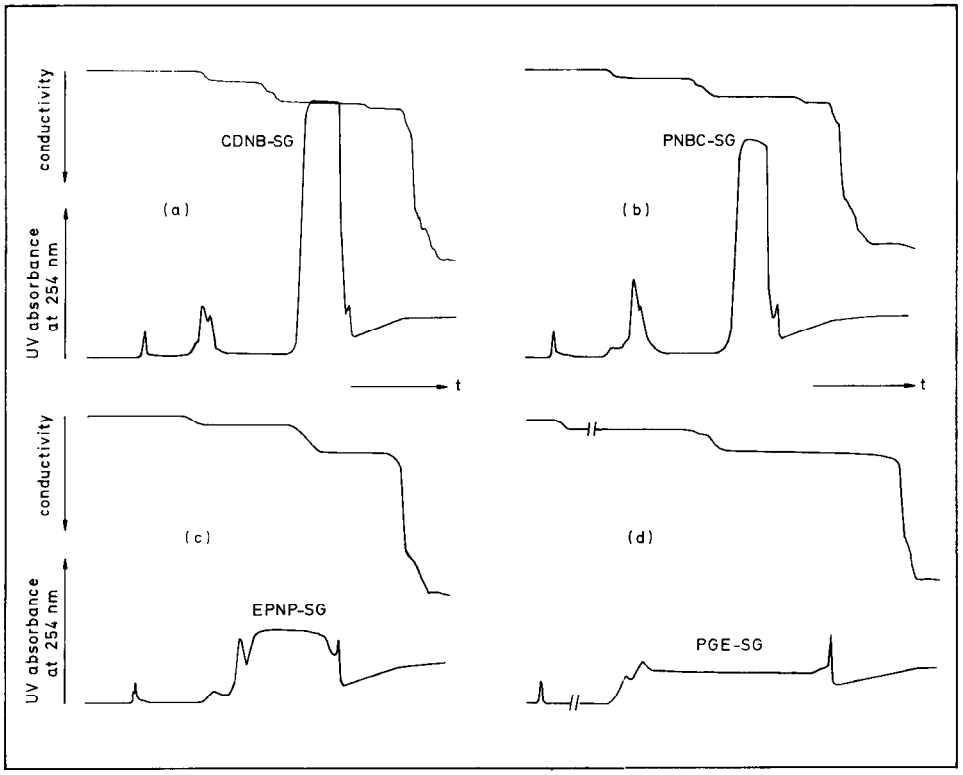

Fig. 3. Isotachopherograms from separate GSH conjugation of (a) CDNB, (b) PNBC, (c) EPNP and (d) PGE in the presence of rat liver cytosolic GST; system A, 23-cm capillary.

was achieved with both systems. Fig. 5a and b show isotachophoretic separations of a mixture of PNBC-SG, PGE-SG and EPNP-SG carried out by using the two electrolyte systems. With both systems a separation of this ternary mixture of GSH conjugates was achieved. The peaks of the GSH conjugates appeared in the same order of migration in both systems (Figs. 4 and 5). Although the GSH conjugates investigated have similar chemical structures, mixtures of at least two conjugates can be clearly separated by using this analytical capillary isotachophoretic method. It was shown that GSH and GSSG in mixtures of GSH conjugates do not disturb the separation of the conjugates. While higher resolution of the GSH conjugates was achieved by using system B, the use of system A led to a more sensitive determination. Differences in resolution between systems A and B as well as between our data and those of Holloway and Battersby[11] may be explained by disturbances from the terminator zone caused by OH^- in our unbuffered terminating electrolyte (high pH).

Generally, GSH conjugates are metabolized to mercapturic acids and excreted in urine or bile after further metabolism in the kidney. Therefore, it was of interest to investigate whether capillary isotachophoresis also allows the determination of mercapturic acids, which are GSH derivatives. Fig. 6 shows an isotachopherogram obtained after complete reaction of 1 m*M* CDNB with a ten-fold molar excess of

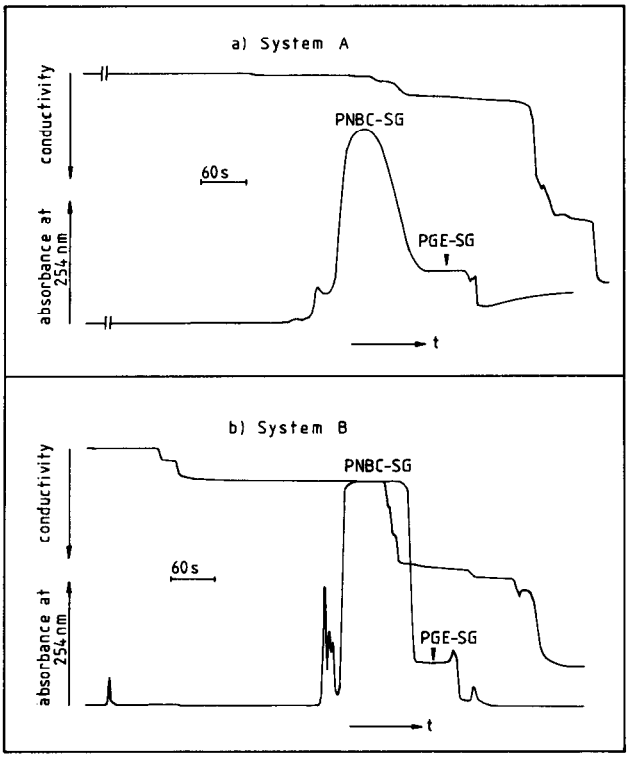

Fig. 4. Isotachopherograms from the separation of a mixture of PNBC-SG and PGE-SG obtained in the electrolyte systems A (a) and B (b).

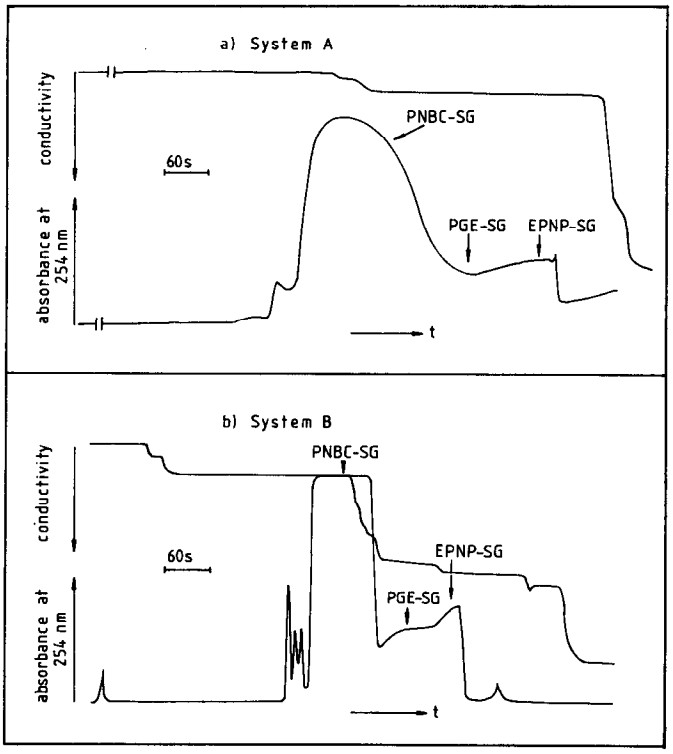

Fig. 5. Isotachopherograms from the separation of a mixture containing PNBC-SG, PGE-SG and EPNP-SG obtained by using the electrolyte systems A (a) and B (b).

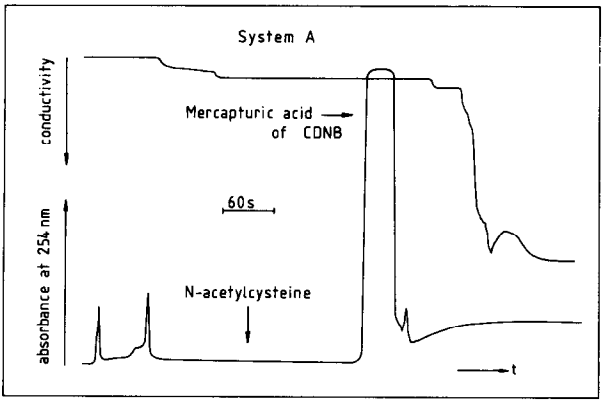

Fig. 6. Isotachopherogram from an incubation of 1 m*M* CDNB with 10 m*M* N-acetylcysteine in alkaline solution after complete reaction.

N-acetylcysteine in alkaline aqueous solution. The mercapturic acid of CDNB appears in the isotachopherogram as a strongly UV-absorbing peak. An addition of GSH, GSSG and CDNB-SG to this mixture resulted in a complete separation of the mercapturic acid from these substances.

The method presented has been applied to the study of reactions for which GSH is a substrate. In Fig. 7 the kinetic profile derived from the isotachophoretic analysis of the enzymatic GSH conjugation of SE at different pH values of the incubation mixture is given. For simplicity, concentration–time courses only of the SE-SG are shown. The decrease in GSH concentration was nearly proportional to the increase of SE-SG. Throughout the incubations the amount of GSSG formed from GSH by autooxidation was negligible (<5%). The higher the pH the higher is the enzymatic and non-enzymatic (not shown) GSH conjugation rate of SE. At pH 9.2 the enzymatic and non-enzymatic reaction rates are equal. This results from the high reactivity of SE and the strong nucleophilicity of the SH group of GSH at high pH values (pK_a SH = 9.2). The pH optimum for the enzymatic GSH conjugation of exogenous and endogenous epoxides was found to be 8.2–8.5[14]. The application of this isotachophoretic method to the determination of GSH stability in various complex media was previously described[14].

The reproducibility and accuracy of the method were checked by repeated isotachophoretic analysis of standard solutions of GSH and EPNP-SG with both electrolyte systems. The specific zone lengths (s/nmol) for GSH obtained from analyses on different days were (mean ± S.D.) 10.38 ± 1.12 for system A (n = 8) and 6.62 ± 0.48 for system B (n = 6). The specific zone lengths (s/nmol) for EPNP-SG determined by injections on different days were found to be (mean ± S.D.) 11.96 ± 0.384 for system A (n = 4) and 7.36 ± 0.45 for system B (n = 4). The standard deviations for GSH and EPNP-SG determined within the same day were of the order of ±0.2.

The sensitivity of the method was sufficient for the accurate detection of 0.1 nmol GSSG, 0.15 nmol GSH, CDNB-SG and PNBC-SG respectively and 0.2 nmol EPNP-SG, when the electrolyte system A was used. The sensitivity could be drastically increased if fluorescence detectors were used. Furthermore, since isotachophoretic

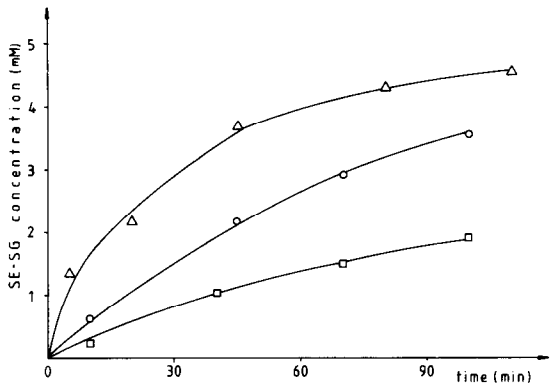

Fig. 7. Kinetic profile derived from the enzymatic GSH conjugation of SE at pH 7.4 (□), 8.5 (○) and 9.2 (△).

analysis of glucuronidation, sulphation and GSH conjugation reactions is possible with the same electrolyte system (system B), we have now started to investigate the applicability of this analytical technique to the simultaneous detection of glucuronides, sulphates and GSH conjugates.

CONCLUSION

The present capillary isotachophoretic method allows a rapid, sensitive and simultaneous determination of at least two GSH conjugates, GSH and GSSG without the need for radioactively labelled compounds or separate analytical procedures. This method also permits the simultaneous determination of GSH conjugates and the corresponding mercapturic acids. The major advantage of this analytical method is its general applicability to the study of reactions involving GSH as substrate.

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