High-Resolution Capillary Zone and Gel Electrophoresis of Structurally Similar Amphipathic Glutathione Conjugates Based on Interaction with β-Cyclodextrins

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The tripeptide glutathione is a prominent intracellular constituent that provides protection against genotoxic and carcinogenic electrophiles and is also a component of several biological signal substances. Glutathione conjugates, free glutathione, and glutathione disulfide contain charged amino acid residues, which contribute to solubility in aqueous media. However, the amphipathic nature of glutathione conjugates and the small differences that may distinguish the S *substituents, pose analytical problems in their resolution. The present study demonstrates how homologous* S*-alkyl and* S*-benzyl conjugates of high structural similarity can be efficiently resolved by capillary electrophoresis. Inclusion of* β*-cyclodextrins in the buffer or in a polyacrylamide gel affords baseline separation of the analytes. The separation methods described are applicable to enzyme assays in vitro and to the identification and quantification of glutathione conjugates of importance in toxicology and physiology. The contribution of* β*-cyclodextrin to the separation is primarily based on interactions between its hydrophobic cavity and the* S*-alkyl and* S*-benzyl groups of the analytes.*

KEYWORDS:

cyclodextrins · electrophoresis · glutathione conjugates · glutathione transferase · peptides

Introduction

Glutathione conjugation is a major pathway in the biotransformation of organic electrophiles of xenobiotic and endobiotic origins.[1] The sulfhydryl group of the tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) reacts with the electrophilic center of the target molecule to form a chemical bond that in many cases is stable in the further metabolism and disposition of the molecule. These genotoxic and potentially carcinogenic agents include activated alkenes, epoxides, and quinones, which can all be formed in the biotransformation of xenobiotics and in the metabolism of endogenous biomolecules.[2] Examples of industrial compounds that could give rise to the glutathione derivatives are alkyl and benzyl halides, which are used in the present investigation. For example, the reaction between benzylbromide and glutathione produces *S*-benzyl-glutathione. Numerous target molecules are genotoxic carcinogens and their reactions with glutathione usually convert them into nontoxic and more water-soluble derivatives suitable for excretion from the cell. Membrane-bound transport proteins affect the export of glutathione conjugates from the cell, and in mammals subsequent metabolic reactions give rise to mercapturates (*N*-acetyl-*S*-substituted cysteine derivatives), which may be excreted in the urine. The conjugation of glutathione with electrophiles is catalyzed by glutathione transferases (GSTs), which differ in their substrate specificities.[3] These enzymes are abundant in mammalian liver and most other tissues and play a prominent role in cellular protection against toxic compounds.[4]

The numerous glutathione conjugates of endogenous compounds are particularly noteworthy from a physiological perspective.[2] Several bioactive molecules arise in eicosanoid metabolism and react with glutathione. Arachidonic acid gives rise to leukotrienes, and the glutathione conjugate LTC_4 and its further metabolites are mediators of the clinical symptoms of asthma. Other bioactive glutathione conjugates derived from polyunsaturated fatty acids are known and new examples are being discovered. Lipid peroxidation of biological membranes produces reactive aldehydes and alkenes, among which 4-hydroxynonenal is known to have chemotactic properties and promote apoptosis.[5] Glutathione conjugation is a prominent biochemical transformation of these oxidation products. *o*-Quinones that arise from dopamine and other catecholamines are possible causative agents of Parkinson's and other degenerative diseases. In all these cases glutathione conjugation is a protective mechanism.[6]

In spite of the biological significance of glutathione conjugation reactions, general, rapid, and efficient procedures for the analysis of glutathione conjugates have not been developed.

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Commonly used GST assays are primarily based on spectrophotometric methods that depend on chromophores attached to a particular substrate.[7] However, many native glutathione conjugates of toxicological interest, such as pesticides and environmental pollutants, do not have the spectroscopic properties necessary for such assays. Therefore, a more universal method is required to monitor the enzymes and to rapidly determine the concentration of the product at high precision, independently of the nature of the substrate and also when it occurs in minute amounts and is not linked to a chromophore. The method would be even more attractive if it also allowed simple and fast spectrophotometric identification of the analytes.

As well as high-performance liquid chromatography,[8] capillary free-zone electrophoresis has successfully been employed to separate GSH and other related compounds in uncoated[9] and coated capillaries[10] and to determine their concentrations.[9c, 10a, 10c] In some cases, underivatized glutathione was separated and detected at short UV wavelengths.[9a, 9b, 9d, 10] Other methods include chemical modification of the substrate, such as labeling with a fluorophore[9c, 11] or Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)),[9a, 10a] to lower the limit of detection (LOD). However, chemical modification has some obvious inherent disadvantages. Therefore, we have investigated whether, 1) the light absorption in the wavelength range 195 – 230 nm permits the detection of substrate and product with the sensitivity and selectivity required, and 2) fast on-line recording of the spectra of the peaks could facilitate the identification of the analytes.

Simple theoretical considerations indicated that the resolution is low for homologues of *S*-alkyl and *S*-benzyl derivatives of GSH in carrier-free buffer solutions. Therefore, we also aimed to find an agent that could form complexes with these homologues such that their resolution increased. Hydroxypropyl-β-cyclodextrin (HP-β-CD), 6-amino-β-cyclodextrin (amino-β-CD), and 2-hydroxy-3-allyloxy-propyl-β-cyclodextrin (allyl-β-CD) appeared to have this property. The last of these derivatives was used as a cross-linker in a polyacrylamide gel. We have previously shown that β-cyclodextrin can affect the mobilities of peptides and proteins.[12]

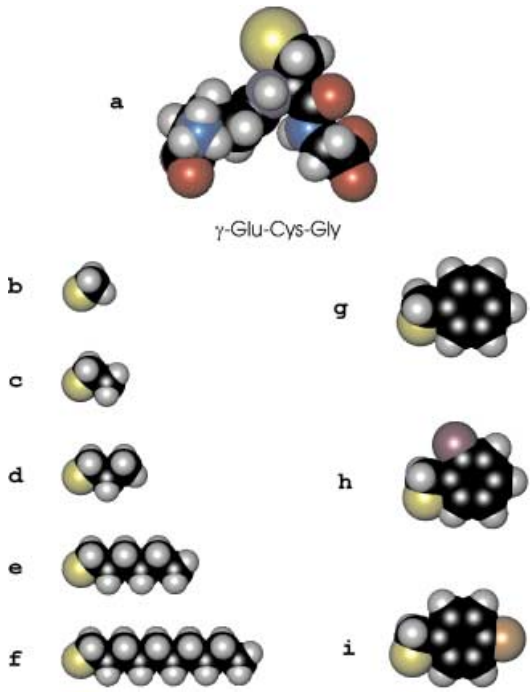

Figure 1. *Structures of glutathione (a) and the alkyl (b – f) and benzyl (g – i) S substituents of the glutathione conjugates analyzed. The yellow, purple, and brown spheres represent sulfur, iodine, and bromine atoms, and the black and grey spheres the carbon and hydrogen atoms, respectively.*

Results and Discussion

The separation mechanism

The electrophoretic mobility (u) of a substance is governed by Equation (1), in which q is the charge of the substance, D is the diffusion coefficient, k is the Boltzmann constant, and T is the absolute temperature (this equation is only strictly valid for mobilities extrapolated to zero ionic strength).

$$u = \frac{qD}{kT} \quad (1)$$

S-Alkyl and *S*-benzyl conjugates of glutathione were chosen as model compounds (for structures, see Figure 1). One can conclude from Equation (1) that for a series of closely related homologues of these conjugates the difference in their diffusion coefficients is too small to permit a satisfactory resolution (see Longsworth's studies of diffusion coefficients of peptides).[13] However, the resolution might be improved if one could find an agent capable of selectively interacting with the benzyl and alkyl groups. We chose β-cyclodextrin as a complexing agent, since it is known that aromatic groups interact with the hydrophobic cavity of the cyclodextrin ring.[14] The addition of hydroxypropyl-β-cyclodextrin dramatically increased the resolution as illustrated by comparison of the experiments presented in Figures 2 and 3.

The tentative interaction model outlined in Figure 4 indicates that alkyl groups should also interact with β-cyclodextrin; this was supported experimentally by the much higher resolution of the alkyl conjugates of glutathione in the presence of HP-β-CD (Figure 2d) compared to that in the absence of HP-β-CD (Figure 2a). Figures 2b – d also show that an increase in the concentration of HP-β-CD, at least in the interval 10 – 50 mM, improves the resolution of the alkyl conjugates significantly. An almost baseline separation of all five alkyl compounds (*S*-methyl-, *S*-ethyl-, *S*-propyl-, *S*-hexyl-, and *S*-decyl-glutathione) and underivatized GSH (Figure 2d) was obtained at 50 mM HP-β-CD.

The benzyl conjugates were baseline resolved at 10 mM HP-β-CD (Figure 3b), but not at higher concentrations (Figures 3c and d), in sharp contrast to the alkyl conjugates (Figure 2b – d). It was somewhat surprising that, 1) the bromo and iodo derivatives were well separated, in spite of the small differences in their diffusion coefficients, and 2) the bromo derivative migrated slower than the iodo derivative even though its molecular mass

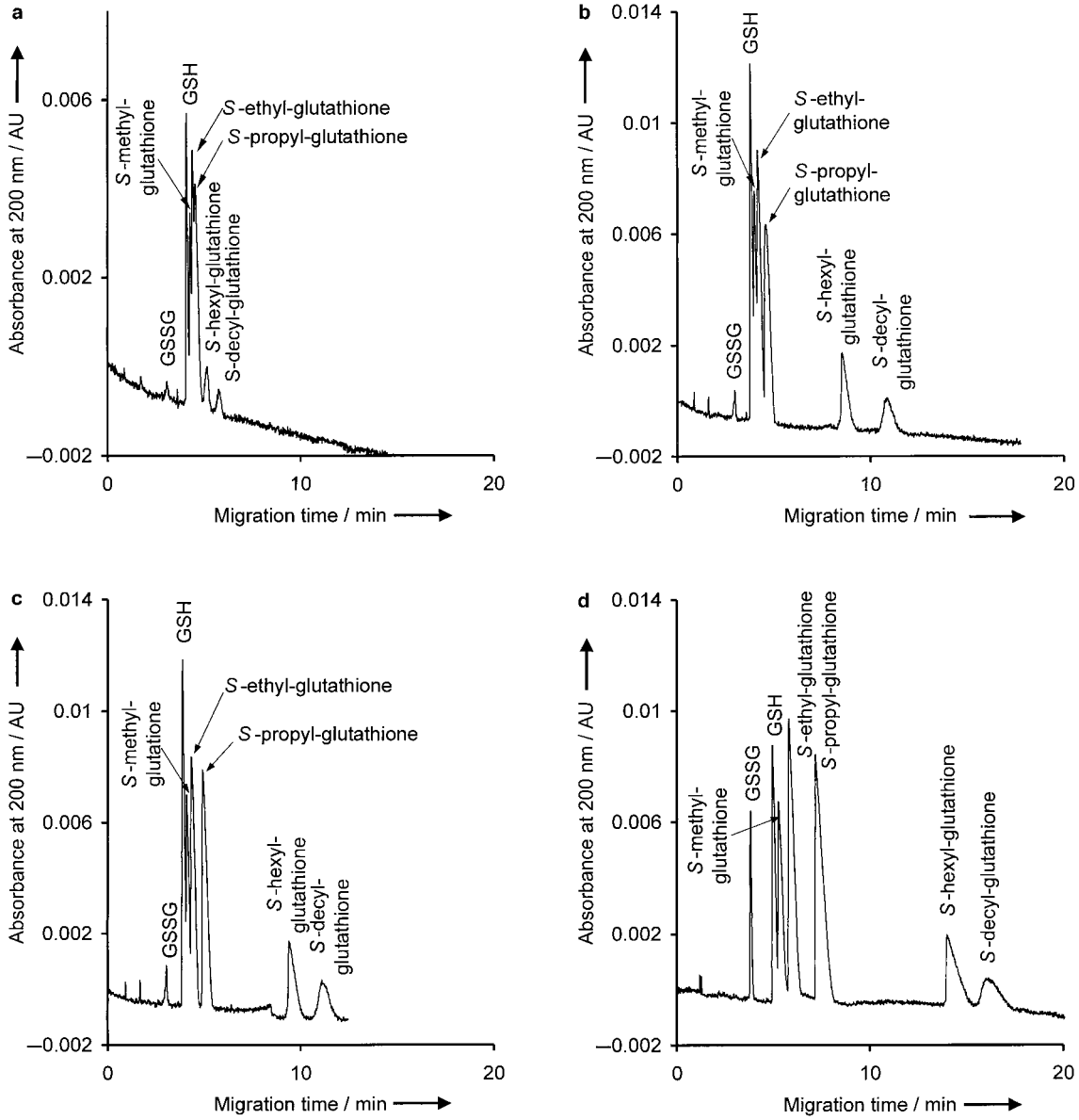

Figure 2. *Electrophoretic separations of S-alkyl-glutathione conjugates in the absence (a) and presence of HP-β-CD at different concentrations (b: 10 mM; c: 20 mM; d: 50 mM). Buffer: 10 mM sodium phosphate, pH 7.0.*

is lower and thus its diffusion coefficient higher [see Eq. (1)]. A tentative explanation could be that because of its somewhat larger "width", the iodo derivative cannot penetrate the cone-shaped cavity of the cyclodextrin molecule (Figure 4) as deeply as the bromo derivative as a result of the difference in the position of the halogen atoms (*ortho* and *para*, respectively).

A plot of t_r/t_0 (t_r = migration time, t_0 = the migration time for the most rapidly moving alkyl conjugate) for the alkyl conjugates against $M^{2/3}/Z$ is linear for the electrophoresis experiment performed in buffer alone, as expected (Figure 5a).[15] The nonlinear relationship and the longer migration times for experiments in buffer that contained HP-β-CD (Figure 5a) are indications that alkyl conjugates also interact with the cyclodextrin ring, as postulated in the models in Figure 4. The separation mechanism for the alkyl conjugates can thus be described in terms of partitioning[16] of the analytes between free buffer and the cavity of the β-CD ring, superimposed on the less pronounced electrophoretic separation (Figure 2a). In partition chromatography, log$k$ ($k = (t_1 - t_0)/t_0$) is proportional to the number of methylene groups in a homologous series of alkyl compounds.[16] A similar relationship should also be approximately valid in electrophoresis where the separation is poor (as in Figure 2a). The plot presented in Figure 5b shows that log$k$ is proportional to the number of methylene groups in the glutathione conjugates for short alkyl chains, which indicates separation of these conjugates occurs based on a partition mechanism. The finding that log$k$ is almost constant for conjugates with six or more methylene groups supports our hypothesis that the cavity of β-cyclodextrin is the moiety involved in the partition because the depth of the cavity is 8 Å,

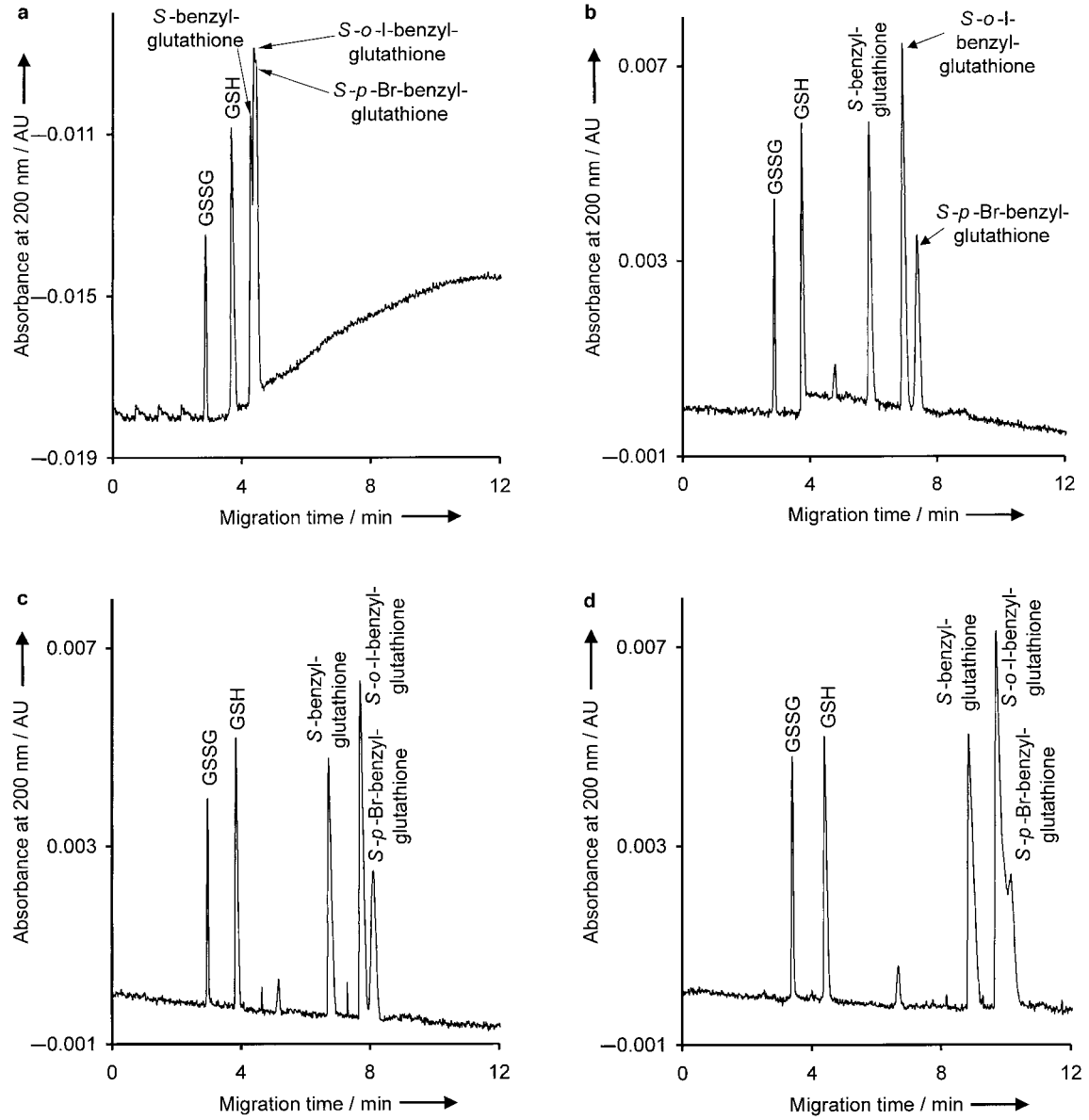

Figure 3. *Electrophoretic separations of S-benzyl-glutathione conjugates in the absence (a) and presence of HP-β-CD at different concentrations (b: 10 mM; c: 20 mM; d: 50 mM). Buffer: 10 mM sodium phosphate, pH 7.0.*

which corresponds approximately to the length of a hexyl residue.

LOD and reproducibility

The lower limit of detection of alkyl conjugates (LOD is assumed to be three times larger than the noise) was 0.7 μM, whereas benzyl conjugates were detectable down to 0.2 μM because of their higher absorption coefficient at 200 nm (the wavelength used for detection). The concentration range tested for linearity was 12 – 620 μM for *S*-methyl-glutathione (equation of regression line: $y = 0.153x$; $R^2 = 0.999$) and 7 – 380 μM for *S*-*o*-iodobenzyl-glutathione (equation of regression line: $y = 0.915x$; $R^2 = 0.999$). The very good linearity over a wide concentration range is noteworthy.

The run-by-run and day-by-day reproducibilities were investigated in a series of analyses of benzyl conjugates (see Table 1) in terms of relative migration times (t_r/t_0). Glutathione was used as an internal reference (t_0), although it was found to interact weakly with HP-β-CD.

Determination of glutathione conjugates in the presence of a large excess of glutathione

The intracellular glutathione concentration may be as high as 10 mM, and the production of conjugates normally involves only a small percentage of the peptide. It is therefore desirable that the product is also detectable in a large excess of glutathione. *S*-Benzyl-glutathione was chosen as a model product for an investigation of biologically relevant conditions. At a concen-

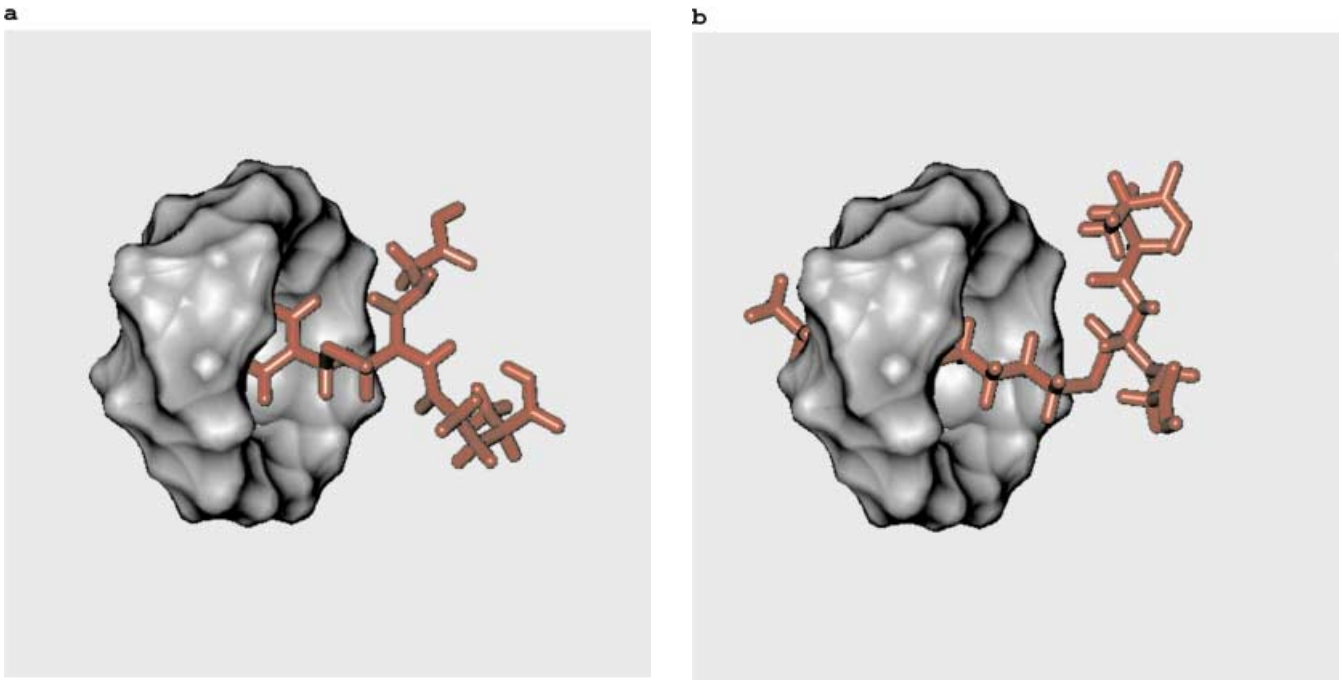

Figure 4. *The possible mode of interaction between the hydrophobic cavity in the cyclodextrin ring and benzyl (a) and alkyl (b) groups. Models of the glutathione conjugates are manually docked into the cavity of the native β-cyclodextrin. The β-cyclodextrin structure was obtained from the Protein Data Bank and is depicted as a surface representation.*

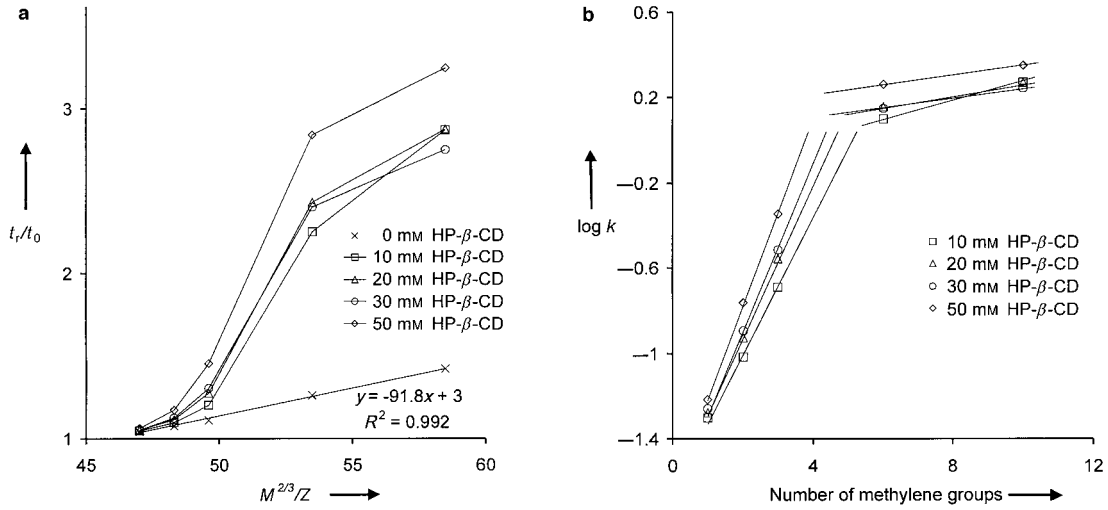

Figure 5. *Plots of a) t_r/t_0 against $M^{2/3}/Z$ and b) log k [$k = (t_r - t_0)/t_0$] against the number of methylene groups in the alkyl conjugates. Log k values were calculated from the migration times in the experiment presented in Figure 2 a.*

Table 1. *Reproducibility of the relative migration times (t_r/t_0) of benzyl conjugates in capillary electrophoretic experiments.*

$n = 5$	r	t_r/t_0[a]	CV [%][b]
run-to-run	*S*-benzyl-glutathione	1.68	0.59
	S-*o*-iodobenzyl-glutathione	2.02	0.66
	S-*p*-bromobenzyl-glutathione	2.12	0.82
day-to-day	*S*-benzyl-glutathione	1.67	1.30
	S-*o*-iodobenzyl-glutathione	2.01	1.05
	S-*p*-bromobenzyl-glutathione	2.11	1.15

[a] The relative migration times ($t_r - t_0$) of the benzyl conjugates were measured in five runs each day (run-to-run) and for five consecutive days (day-to-day); t_0 = migration time of GSH, t_r = migration time of conjugate. Values are given as mean values. [b] CV = coefficient of variation.

tration of 20 μM, this conjugate could be separated from GSH even when the molar concentration of GSH (3 mM) was 150-fold higher than that of the conjugate (Figure 6 a). In the presence of HP-β-CD (50 mM), the resolution was significantly improved (Figure 6 b).

The electropherograms show that these two compounds will also be baseline separated in enzyme assays, in which their concentrations may be considerably higher.

Comparison between nonenzymatic and enzymatic reactions

GSH and an electrophilic substrate can react nonenzymatically. Therefore, the contribution of this background reaction to the observed separation pattern in the presence of enzyme must be

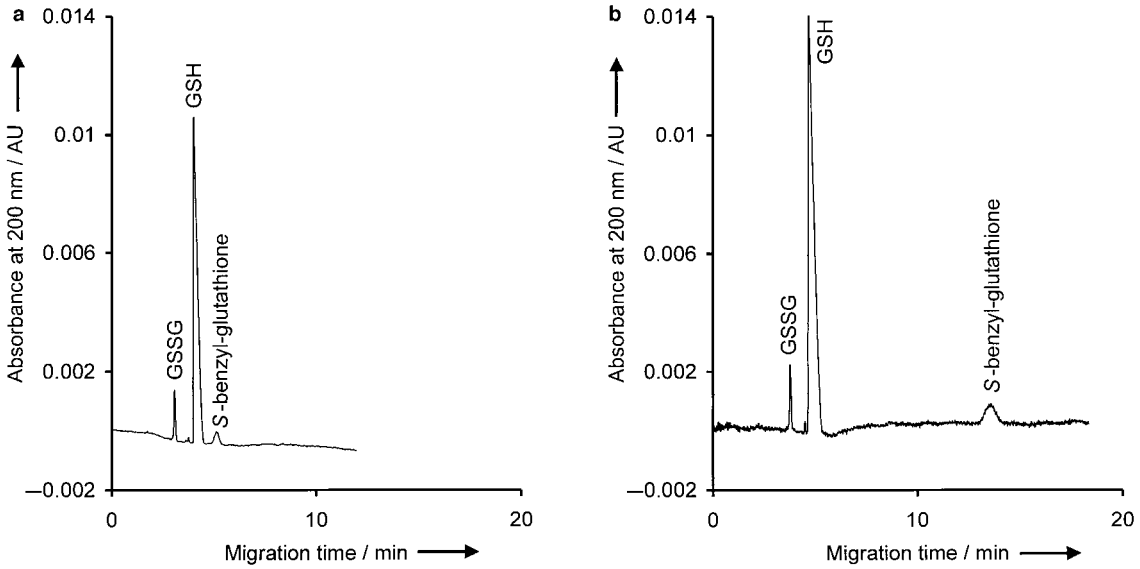

Figure 6. Electrophoretic separation of S-benzyl-glutathione from a 150-fold molar excess of glutathione. Capillary free-zone electrophoresis in the absence (a) and presence (b) of 50 mM HP-β-CD. Buffer: 10 mM sodium phosphate, pH 7.0.

known and subtracted from the measured reaction to determine the true enzyme activity. 1-Chloro-2,4-dinitrobenzene (CDNB) was employed to investigate the background reaction, since it is a common substrate for studies of glutathione transferase and is known for its rapid nonenzymatic reaction with glutathione.[7] CDNB is neutral and therefore does not migrate electrophoretically in a coated capillary. GSH (1 mM) was mixed with CDNB (1 mM). Then electrophoretic separations of these compounds were performed in free solution in a coated capillary in the absence of the enzyme and buffer additives. The conjugate formed by CDNB and GSH had a lower electrophoretic mobility than unconjugated GSH. Therefore, the peak of the conjugate followed the peaks of glutathione disulfide (GSSG) and GSH. The UV spectrum of the conjugate differed from those of these molecules in that the conjugate absorbed light in the entire UV range with a maximum at 340 nm. The areas of the peaks at 200 nm (A_{GSH} and A_{conj}) were determined by integration and the ratio $A_{GSH}/(A_{GSH} + A_{conj})$ was plotted against the incubation time (Figure 7, curve I). A similar series of runs was performed with a GSH/CDNB mixture, to which human glutathione transferase P1-1 (final concentration 28.5 ng mL^{-1}) was added (Figure 7, curve II). The progression of the reaction is considered to have stopped after application of the electrical field, since the time that the substrates are in contact with each other in the capillary is negligible before they separate.

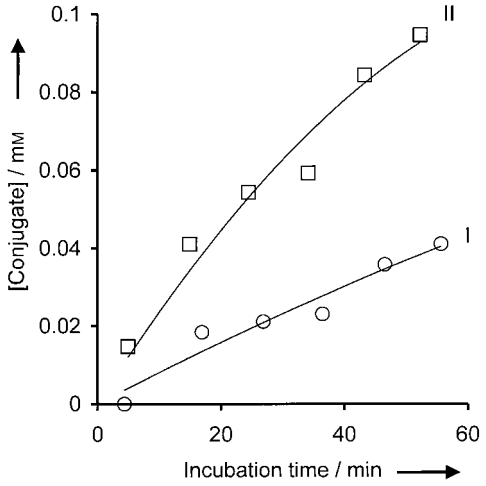

Figure 7. Comparison of the nonenzymatic background (I) and the enzymatic (II) reactions between glutathione and 1-chloro-2,4-dinitrobenzene. Reactions were carried out at 20 °C in 0.01 M sodium phosphate at pH 7.0, with the reactants at 1-mM concentration. The conjugation reaction was terminated at different times for a series of aliquots injected into the capillary for electrophoresis.

Capillary electrophoresis in buffers containing positively charged β-cyclodextrin (6-amino-β-cyclodextrin)

The presence of uncharged HP-β-CD in the background electrolyte improved the resolution obtained (Figures 2 and 3). Therefore, it was logical to investigate the separation capability of a β-cyclodextrin with a charge opposite to that of the analytes. As expected, the positively charged 6-amino-β-cyclodextrin (amino-β-CD) gave longer migration times because the complexes formed between the amino-β-CD and the conjugates were less negatively charged than those formed with neutral β-CD (compare Figures 8a and 3b). The resolution was significantly improved, which suggests that different types of interactions are involved in complex formation with amino-β-CD from those involved in β-CD separations. Electrostatic attraction between the amino group on the cyclodextrin and carboxylic groups on the conjugates does not appear to play an important role, since the electropherogram for the amino-β-CD experiment (Figure 8a) is similar to that obtained in a neutral cyclodextrin–polyacrylamide gel (Figure 8b). This result is further discussed in the next section.

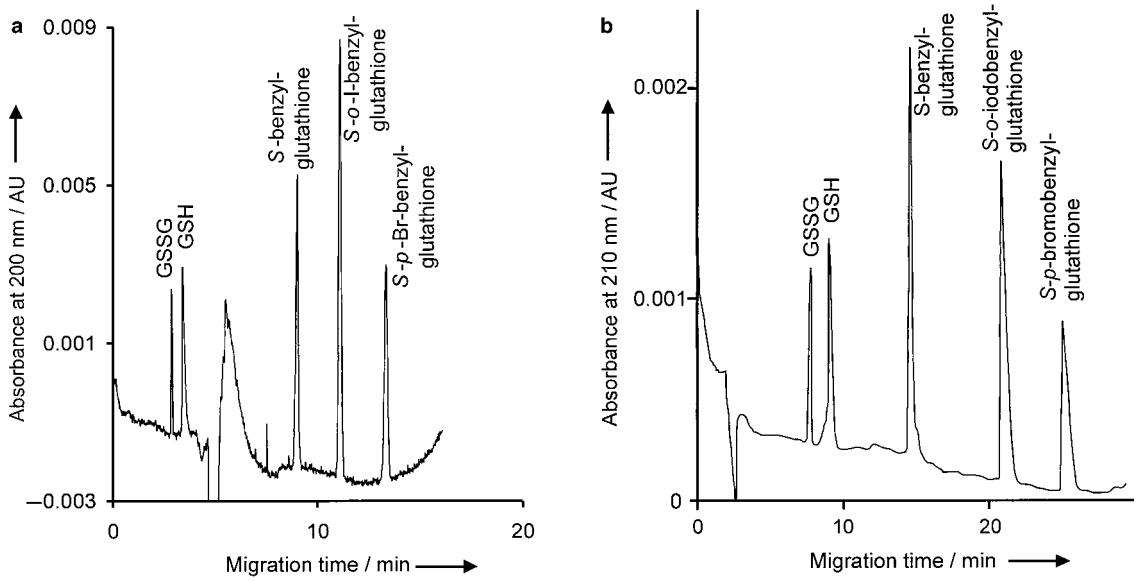

Figure 8. *Separation of benzyl conjugates (a) in the presence of 6-amino-β-cyclodextrin (10 mM) in free buffer (10 mM sodium phosphate, pH 7.0) and b) in acrylamide gel (3%) cross-linked with allyl-β-CD (10 mM) in the absence of amino-β-CD (buffer: 0.1 M Tris/0.15 M borate, pH 8.2).*

Capillary electrophoresis in gels containing β-cyclodextrin derivatives

Very high resolution of the conjugates similar to that achieved in free solution in the presence of charged amino-β-CD (Figure 8a) was obtained by using an uncharged acrylamide-based gel cross-linked with a highly substituted derivative of β-CD, 2-hydroxy-3-allyloxy-propyl-β-cyclodextrin (allyl-β-CD; Figure 8b). It is well known that most analytes can interact with chromatographic matrices[17] and such interactions are also characteristic of acrylamide gels.[15, 18] Aromatic groups in particular, interact with polymers and gels. This so-called "aromatic adsorption"[17] has been exploited in electrophoresis,[15] chromatography,[17, 18] and electrochromatography.[19] The strong retention of the benzyl derivatives used in this study may be ascribed to this type of adsorption, in addition to the interaction with the cavity of cyclodextrin and other weak interactions (also between hydrophilic moieties).

The electropherograms obtained in carrier-free experiments conducted in a tris(hydroxymethyl)aminomethane (Tris)/boric acid buffer (pH 8.2) were very similar to those performed in a sodium phosphate buffer (pH 7.0) (results not shown). Accordingly, the large differences between the separation patterns observed in gel (Figure 8b) and free-buffer (Figure 3a) experiments cannot be explained by differences in the background electrolytes (see Table 2), but is rather a result of the above-mentioned solute interactions with the gel. The advantage of the Tris/borate buffer is that it permits higher field strength because of its lower electrical conductivity, which results in shorter analysis times without generation of thermal zone deformation, which was observed with the phosphate buffer.

The influence of allyl-β-CD on the separation mechanism should be similar in experiments in gel and in free solution, that is, separation should be independent of whether the cyclodextrin molecule is immobilized in the gel or added to the buffer. We want to stress that an increase of the gel concentration to 50 mM (results not shown) caused, 1) baseline separation of the alkyl conjugates, 2) such strong retardation of the *S*-hexyl- and *S*-decyl-glutathiones that they did not pass the detection window, even upon prolonged run time, and 3) co-migration of *S*-*o*-iodobenzyl- and *S*-*p*-bromobenzyl conjugates (compare Figures 3a and d).

Table 2. *Mobilities of glutathione in various electrophoretic media.*

Medium	Mobility [T unit][a]
10 mM sodium phosphate, pH 7.0	−18
10 mM sodium phosphate, pH 7.0 + 10 mM HP-β-CD (final pH = 7.0)	−18
10 mM sodium phosphate, pH 7.0 + 10 mM amino-β-CD (final pH = 8.4)	−20
0.1 M Tris/0.15 M boric acid, pH 8.2	−22
0.1 M Tris/0.15 M boric acid, pH 8.2 + 10 mM HP-β-CD (final pH = 8.2)	−21
0.1 M Tris/0.15 M boric acid, pH 8.2 + 10 mM amino-β-CD (final pH = 8.2)	−20

[a] 1 T(iselius) unit = 10^{-5} $cm^2 V^{-1} s^{-1}$.[23]

Conclusion

We conclude from the series of experiments presented that all the tested glutathione conjugates can be separated with good resolution by capillary electrophoresis in the presence of HP-β-CD and can be detected at low concentrations. The HP-β-CD is more water soluble and less expensive than the underivatized β-cyclodextrins. Gels of acrylamide cross-linked with allyl-β-cyclodextrin give similar or higher resolution than HP-β-CD alone

(compare Figures 3b and 8b) although the migration times are longer as a result of restricted migration in the pores of the gel.

Capillary zone and gel electrophoresis in the presence of HP-β-CD, amino-β-CD, and acrylamide/allyl-β-CD gels afford baseline separation of amphipathic glutathione conjugates with similar structures and physical properties. A comparison of Figure 2a and Figure 2b – d reveals that HP-β-CD and particularly amino-β-CD (Figure 8a) should be added to the buffer to improve the resolution. The separations achieved in a gel (Figure 8b) are similar to those obtained in free solution with amino-β-CD as a buffer additive (Figure 8a). The latter medium must be used when the protein components (including the enzyme) in the sample are to be recorded because the gel pores are so small that the migration velocity of proteins through the gel is close to zero. When the protein peaks (partially) coincide with other peaks of interest, the gel alternative may be preferable. Electrophoresis in these narrow-pore gels can, therefore, also be utilized for on-line enrichment of glutathione transferase (or any other charged macromolecule) at the buffer/gel interface upon prolonged electrophoretic injection of a dilute sample. In such experiments, the gel cannot be used more than once or twice because its pores will be clogged. For the separation of small molecules, the lifetime of the gel is often weeks, even upon repeated analyses. We have developed several concentration methods for proteins and low-molecular-weight substances[20] that can be utilized for the sample components used in this study.

In the discussion of the separation mechanism we emphasized that β-CD cannot discriminate between long alkyl chains that differ only by one or two methylene groups. It is likely that reversed-phase methods can resolve these compounds. However, when the difference is larger, alkyl chains can be separated by free-zone electrophoresis in the absence of β-CD by virtue of the differences in the diffusion coefficients [Eq. (1)], as experimentally verified for the hexyl and decyl derivatives (Figure 2a).

It should be remembered that alterations in the ionic strength and the composition of the buffer often change the appearance of an electropherogram, although seldom drastically. When the GSH peak (partially) coincides with another peak and thus makes the analysis inaccurate, its position in the electropherogram can be shifted by changing the pH value of the buffer. The background electrolytes and the additives used in this investigation do not form complexes with GSH and, therefore, are not expected to dramatically affect its mobility; this is confirmed by the data in Table 2.

Alterations in the concentrations of the allyl-β-CD gels have a profound influence on the resolution and should therefore be utilized in attempts to optimize the separation.

Experimental Section

Materials and instruments: Fused-silica tubing was purchased from MicroQuartz (Munich, Germany). Acrylamide, ammonium peroxysulfate (APS), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were from Bio-Rad (Hercules, CA, USA); 3-(trimethoxysilyl)propylmethacrylate (Bind-Silane), β-cyclodextrin (β-CD), and hydroxypropyl-β-cyclodextrin (HP-β-CD) were from Fluka (Buchs, Switzerland); 6-amino-β-cyclodextrin (amino-β-CD) was obtained from Advanced Separation Technologies Inc. (Whippany, NJ, USA); glutathione, glutathione conjugates, and allylglycidyl ether were obtained from Sigma (St. Louis, MO, USA). The enzyme, human glutathione transferase P1-1/Ile 105, was obtained at a concentration of 11.4 mg mL^{-1} and had a specific activity of about 100 μmol min^{-1} mg^{-1}.[21]

A BioFocus 3000 capillary electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA) with fast scanning detection was used for all experiments. This instrument allowed a compound to be identified not only from its *k* value, but also from its spectrum. All experiments in gel-filled capillaries were performed in a home-built instrument based on a Linear Model 200 UV detector (Linear Instruments, Reno, NV, USA).

Capillary free-zone electrophoresis: Free-zone electrophoresis experiments were carried out in fused-silica capillaries (23 cm total length; 18 cm to the detection window). The capillaries (internal and external diameters 50 and 365 μm, respectively) were coated with 5% linear polyacrylamide.[22] Acetone did not pass the detection window during a run time of 80 min, which indicates a very low electroendosmosis. Sample compounds were dissolved in sodium phosphate (10 mM) at pH 7.0 and injected hydrodynamically (1 psi × s). Separations were carried out in 10 mM sodium phosphate buffer at pH 7.0 under an applied voltage of 10 kV (with a negative to positive polarity), which yielded a current of approximately 15 μA. Regeneration of the capillaries between successive analyses was achieved by purging with the running buffer (10 mM phosphate, pH 7.0) for 30 s.

Capillary gel electrophoresis: The 2-hydroxy-3-allyloxy-propyl-β-cyclodextrin (allyl-β-CD) was synthesized by a simple procedure.[19] Acrylamide (30 mg) was dissolved in Tris/boric acid (100 mM/150 mM) buffer (1 mL, pH 8.2) that contained different volumes (100 or 500 μL) of allyl-β-CD (100 mM). These acrylamide/allyl-β-CD solutions (150 mL of each) were degassed with a water pump and 5% (*w*/*v*) APS (5 μL) and 5% (*v*/*v*) TEMED (5 μL) were then added. This solution was sucked immediately into the silanized fused-silica capillary (internal diameter: 50 μm).[22] The polymerization started within a few minutes and was almost complete within half an hour, but the capillaries were usually not employed until later. The gel-filled capillaries could be used repeatedly for long periods of time without bubble formation or loss of resolution. The sample was injected electrokinetically at the cathodic end of the capillary ($l = 14$ cm, $L = 16.5$ cm).

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