ELSEVIER

Journal of Chromatography B, 705 (1998) 251–259

JOURNAL OF CHROMATOGRAPHY B

Comparison of anion-exchange and ion-modified reversed-phase liquid chromatography for the determination of *S*-sulfocysteine

Wanlin Xia[a], Mats Sandberg[b], Stephen G. Weber[a,*]

[a]*Chevron Science Center, University of Pittsburgh, Pittsburgh, PA 15260, USA*
[b]*Institute of Neurobiology, Department of Histology, Göteborgs Universitet, Medicinaregatan 5, Göteborg, Sweden*

Received 5 March 1997; received in revised form 7 October 1997; accepted 16 October 1997

Abstract

A dual Hg–Au amalgam electrode is used to detect *S*-sulfocysteine (SSC) in this study. There exist two main components in the acetonitrile (ACN) rat brain extracts, namely, Cl^- and GSSG (oxidized glutathione), that are active in our detection system (GSH is not extracted in ACN). Two strong anion-exchange columns from different companies were used to separate the samples under different conditions, but SSC and Cl^- were not separated at the optimum detection pH of 5.2. The signal from Cl^- was greatly decreased by lowering the potential at the downstream electrode, though it cannot be completely eliminated. While a silver cartridge removed Cl^- from micromoles to several millimoles without any negative effect on the SSC signal in aqueous standards, a large negative peak which interferes with SSC detection was unfortunately introduced when a silver cartridge was applied to brain tissue samples. However, SSC and Cl^- in the samples are successfully separated by ion-modified reversed-phase LC in acetate buffer at the optimum detection pH (5.2). The separation conditions are 20 m*M* acetic acid, 2% methanol, 0.5 m*M* cetyltrimethylammonium *p*-toluene sulfonate (CTMA) (pH 5.2). Most importantly, the sensitivity of SSC under the optimum separation conditions is not sacrificed. The detection limit is 8 n*M* (20 μl injected).

Keywords: *S*-Sulfocysteine; Thiol; Cysteine

1. Introduction

L-*S*-Sulfocysteine (Fig. 1), L-glutamate and certain other acidic amino acids can cause neuronal degeneration [1]. This so-called excitotoxicity is initiated by overactivation of glutamate-receptors such as the *N*-methyl-D-aspartate (NMDA)-receptor leading to an abnormally high influx of calcium which in turn can start degenerative processes [1–3]. SSC is a highly potent and selective NMDA-receptor agonist [4] but it is not known if endogenous SSC functions as a transmitter and/or excitotoxin in vivo. Another potentially lethal property of SSC is its inhibitory effect on the enzyme γ-glutamylcysteine synthetase [5], the rate-limiting step in glutathione production, a

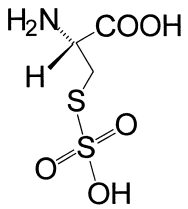

Fig. 1. Structure of L-*S*-sulfocysteine (SSC).

*Corresponding author.

0378-4347/98/$19.00
PII S0378-4347(97)00518-5

property of SSC which will reduce the cell's ability to inactivate free radicals [6]. As a sulfur-containing amino acid, SSC has recently been found to modulate the release of noradrenaline from hippocampal slices [7]. SSC could be a neurotoxic factor in sulfite-oxidase deficiency, a syndrome accompanied by mental retardation and high SSC concentrations in urine and plasma [8,9]. Molybdenum cofactor deficiency is another neuropathological disorder in which high SSC concentrations are found in urine [10]. Systemic injections of cysteine are neurotoxic to neonatal rats [11,12] and prolonged ischemia is accompanied by a dramatic accumulation of cysteine [13] but it is not known if SSC is elevated and involved in the toxicity. In order to be able to study the intra- and extracellular dynamics of endogenous SSC in vitro and in vivo, a selective and sensitive analysis system is needed. The sensitivity of the NMDA receptor to SSC [4] suggests that concentrations in the sub-micromolar to micromolar range might be expected in brain, with higher concentrations resulting from an increase in cysteine concentration. The determination of SSC at low concentrations is challenging because of its highly acidic nature which can result in chromatographic interferences with other endogenous acidic sulfur-containing amino acids such as cysteine sulfinate, cysteate, homocysteine sulfinate, and homocysteate [14]. Being a thioester, SSC is also a relatively unstable compound which makes derivatization involving reducing compounds, such as β-mercaptoethanol in precolumn OPA-derivatization, impossible. Here we have continued our study on the characterization of electrochemical detection in combination with ion-exchange chromatography for SSC separation and detection.

The purpose of our study is to develop an effective method for SSC. Our previous report [15] has shown that the dual Hg–Au amalgam electrode detection system, successfully used by Lunte and Kissinger [16] to detect thiols and disulfides in rat liver extracts, is appropriate for our objective, and further that the optimum detection pH is around pH 5.2. As brain tissue samples have been tried, however, the separation of SSC from interferences has been found to be very challenging. Several reasons account for this problem. In particular, the detection of SSC, as shown in our previous work [15], is highly sensitive to flow-rate, pH and buffer types, which limits the range of choices available to improve the separation.

The majority of the LC methods used to separate biological thiols and disulfides are strong cation-exchange LC with mobile phases in the lower pH range, and reversed-phase LC with mobile phases containing a large hydrophobic anion (i.e. modifier ion) [16–22]. These modes are not appropriate in our case, since SSC is an anion over a wide range of pH, particularly at its optimum detection pH 5.2, due to the low pK_a (1–2) of the *S*-sulfo functional group.

To obtain a reasonable retention time for SSC, both strong anion-exchange LC (SAX) and ion-modified reversed-phase LC (IM-RPLC) with mobile phases including a large hydrophobic cation were investigated. As far as our separation was concerned, IM-RPLC was more powerful and selective than SAX. At the same time, it was found that the separations achieved by IM-RPLC were highly dependent on buffers and ion-modifying reagents.

2. Experimental

2.1. Chemicals

L-*S*-Sulfocysteine cysteine, glutathione (GSH), and oxidized glutathione (GSSG) were purchased from Sigma (St. Louis, MO, USA) and used without further purification. The ion-modifying agents, tetrabutylammonium perchlorate (TBA) and cetyltrimethylammonium *p*-toluenesulfonate (CTMA) were also bought from Sigma. Triply distilled mercury was from Bethlehem Apparatus Co. (Hellerton, PA, USA). Water for all studies was prepared by a Milli-Q purification system (Millipore; Bedford, MA, USA). Malonic acid, bought from Fisher (Fair Lawn, NJ, USA), was recrystallized in doubly deionized water. All other compounds used in this study were AR grade or better and bought from commercial sources.

2.2. Instrumentation

Electrochemical detection was completed with a dual Hg–Au amalgam electrode detection system, described in our previous work [15], in which a potentiostat LC-4B (BAS) and a thin-layer cell

(BAS) were used. A flow cell with about a 20-μl volume was produced by using two Teflon spacers of 0.005 inch from BAS. A reference electrode, Ag/AgCl (3 *M* NaCl), was used in all experiments. While the potential of the upstream electrode was set at −1.5 V, the potential at the downstream electrode was adjusted to around 0.0 V to maintain the background current close to zero. Only the downstream electrode was monitored. A Waters pump, Model 600-MS, was used for separations. In SAX, a syringe pump (ISCO, Model 100 DM) was sometimes used to adjust the post-column pH of the mobile phase. The column temperature was controlled by the LC-22 Temperature Controller from BAS. The software for data collection and analysis was EZChrom from Scientific Software, which was installed in a Micron P-75 PC; an SS420 A/D board was used. Injection was accomplished by a Rheodyne LC injector equipped with a 20-μl injection loop. All the buffer pH values were adjusted with aqueous sodium hydroxide to the desired values, which were recorded by a pH meter (Accumet) from Fisher Scientific. Mobile phases were continuously degassed with helium to exclude air.

Two kinds of microbore SAX columns were used. One was a Zorbax-NH_2 column (5 μ*M*, 250×1 mm) with a 20×1 mm guard column of the same material (Micro-Tech Scientific, Inc.) and the other one was a Nucleosil SB column (5 μm, 250×1 mm) with a 20×1 mm Nucleosil SB guard column, which was purchased from Keystone, Inc. For IM-RPLC chromatography, a Zorbax XDB-C8 column (5 μm, 150×2.1 mm), bought from MAC-MOD Analytical, Inc., was used without a guard column. Before recording data in IM-RPLC, the column was stabilized by pumping mobile phase containing ion-modifying reagent for at least 12 h. All mobile phases were filtered through a 0.45-μm Nylon filter.

2.3. Elimination of chloride ion

In this study, efforts have been made to remove Cl^- from samples. The device includes a disposable, plastic 1-ml syringe from Aldrich, a silver cartridge from Dionex and a filter with 0.5-μm pore size from Millipore. The Ag^+-cartridge was a cation-exchange resin in the Ag^+ form. This cartridge was used to remove Cl^- from samples by forming and sorbing silver chloride. Samples were taken with a syringe and then ejected through the Ag^+-cartridge and the filter. The flow-rate was controlled at about 1 ml/min by hand, which can be estimated by the dropping rate. The first several drops (about 200 μl) were not collected.

2.4. Preparation of standards

Standards of SSC, sodium chloride, cysteine, cystine, GSH and GSSG were prepared in doubly deionized water. All the samples were stored at −20°C.

2.5. Preparation of rat brain samples

Neonatal rats (4d) were decapitated and part of the cerebral cortex (20–40 mg) was sonicated and left to stand in ACN for 12 h. Following centrifugation (13000 *g*×30 min), a clear supernatant was taken and evaporated to dryness in a Speedvac (a centrifuge coupled to a vacuum pump). Before analysis, the brain extracts were dissolved in doubly deionized water, centrifuged (800 *g*) for about 10 min and filtered through a 0.45-μm filter.

3. Results and discussion

3.1. Anion-exchange LC

A typical chromatogram of rat brain extract and standard SSC obtained from SAX around pH 5.2 is shown in Fig. 2. As SSC is an anion over a wide range of pH, SAX was first explored to separate the rat brain extracts. Because of the selectivity of the acetonitrile extraction used for the neonatal rat brain tissues and the detection system, the extracts show only two main peaks, which were attributed to Cl^- and oxidized glutathione (GSSG) by comparing their retention times with those from standard NaCl and GSSG in this anion-exchange chromatographic mode. However, the Cl^- peak interferes with the standard SSC peak. Therefore, the challenging problem to be solved is to separate SSC from Cl^- effectively.

Two different kinds of strong anion-exchange columns, namely, Zorbax-NH_2 column and Nu-

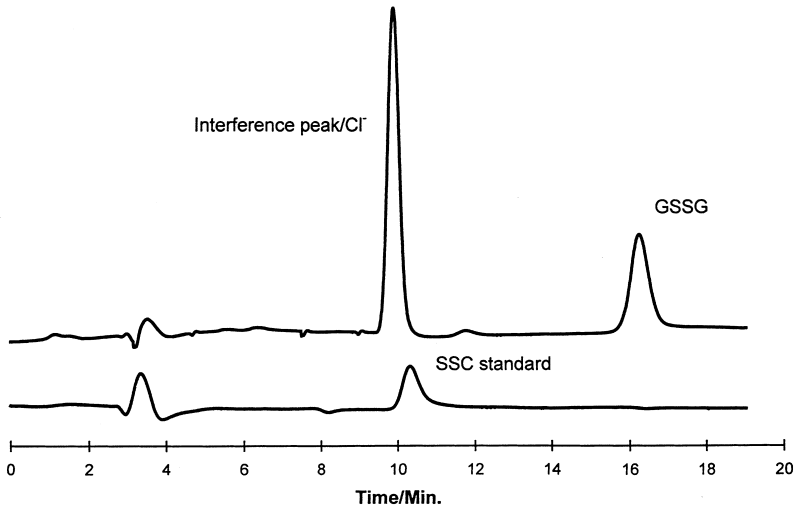

Fig. 2. Typical chromatograms of rat brain extract and *S*-sulfocysteine from strong anion-exchange LC. Mobile phase: 20 m*M* malonate buffer (pH 5.2) including 5% methanol. Column: strong anion-exchange Nucleosil SB (250×1 mm, 5 μm).

cleosil-SB, gave very similar results, with only minor differences in the retention times of SSC, Cl^- and GSSG. As seen from Fig. 2, the huge interference peak (Cl^-) almost overlapped the SSC peak. While many factors can influence the retention in ion-exchange chromatography, the main parameters include mobile phase pH, concentration of electrolyte, temperature and type of buffer salt [23]. In SAX, buffer salt effects are usually adjusted by changing the mobile phase anion. This approach was unfortunately limited in our study, since the common anions used, such as SO_4^{2-}, Cl^-, F^-, Br^- and I^-, are not appropriate because they will react with mercury and influence the detection system. Simply, changing the mobile phase pH can often change the separation pattern [24]. In this case, the resolution of Cl^- and SSC in citrate buffer varied from 0.1–0.95 over the pH range of 3.5–6.5, with the poorest resolution at the optimum detection pH.

Other factors, including concentration of organic modifier, buffer type, separation temperature and ionic strength in the mobile phase, were therefore explored. The buffer system that causes the fewest problems for the detector is citrate, whereas malonate and monochloroacetate mobile phases cause some electrode fouling. The results in three different buffers under typical conditions are given in Table 1. The best chromatographic separation was achieved in monochloroacetate buffer at pH 3.0. This buffer does not work near the best pH for detection (pH 5.2) due to its low pK_a (2.85). Thus a second pump was used to adjust the mobile phase pH to pH 5.2 before detection. A concentrated buffer, 500 m*M* citrate (pH 5.5) including 5% methanol, was initially used for this second solution. Despite the mobile phase being at the optimum pH, the sensitivity to SSC was very low and the detection limit was estimated to be about 5 μ*M*. By decreasing the buffer concentrations from 40 m*M* and 500 m*M* to 10 m*M* monochloroacetate (pH 3.0) and 50 m*M* citrate (pH 5.8), respectively, with flow-rates of the two phases and the final pH after mixing unchanged (around pH 5.2), the sensitivity of SSC was increased. A detection limit of around 0.2 μ*M* was obtained. This dramatic sensitivity to detection conditions makes it essential to find better separation conditions under which the sensitivity of SSC is also acceptable.

3.2. Elimination of Cl^- before anion-exchange LC

Since the interference is mainly from Cl^-, the separation problem would be solved if the signal from Cl^- could be decreased. The signals from Cl^- and SSC depend on the downstream electrode potential as shown in Fig. 3. As the potential decreased from −0.04 V to −0.08 V, the signal of Cl^-

Table 1
Resolution of Cl^- and SSC peaks in SAX

pH	Buffer	Other salt	CH_3OH (v/v)	CH_3CN (v/v)	T (°C)	Resolution
3.0	10 m*M* $ClCH_2COOH$	5 m*M* $NaClO_4$	5%	0	22	0.95
3.0	40 m*M* $ClCH_2COOH$	None	5%	0	22	1.0
3.6	10 m*M* citric acid	None	5%	0	22	0.95
3.6	10 m*M* citric acid	None	0	5%	22	0.0
5.2	10 m*M* citric acid	None	5%	0	22	0.08
5.2	10 m*M* citric acid	None	5%	0	50	0.18
5.2	10 m*M* citric acid	None	0	5%	22	0.0
5.2	20 m*M* malonic acid	None	5%	0	22	0.27
5.2	20 m*M* malonic acid	5 m*M* $NaNO_3$	5%	0	22	0.57

decreased significantly whereas that of SSC remained almost unchanged. However, at −0.12 V, a decline in the response of SSC and baseline shifting became serious. Unfortunately, the Cl^- peak could not be completely removed just by lowering the working potential.

Another choice to eliminate the signal from Cl^- is to use silver nitrate to precipitate it. However direct use of silver nitrate to samples is not practically feasible, since the amount of Ag^+ is hard to control and even a little excess Ag^+ will contaminate the electrode. Fortunately, the Ag^+-cartridge seems to be able to overcome this problem. Both standard Cl^- and SSC were exposed to the Ag^+-cartridge. It was observed (data not shown) that the Ag^+-cartridge can remove Cl^- very effectively over a wide range of concentrations (micromoles to several millimoles). Most importantly, this silver cartridge has no obvi-

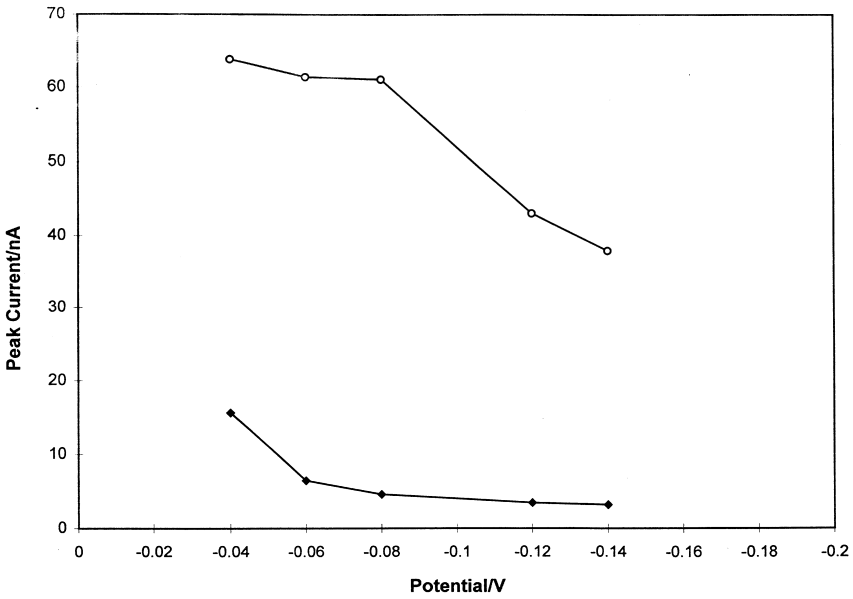

Fig. 3. Dependence of the responses of Cl^- and SSC on detection potentials. Mobile phase: 10 m*M* citrate (pH 5.2) including 5% methanol. Flow-rate: 0.05 ml/min. The same anion-exchange column as in Fig. 2 was used. Lower curve (♦): 20 μl of 50 μ*M* Cl^- were injected for each point. Upper curve (○): 20 μl of 50 μ*M* SSC were injected for each point.

ous negative effect on the sensitivity of SSC in aqueous standards. Unfortunately, when brain tissue samples were used, an additional large negative peak was formed. The negative peak overlapped with the SSC peak and made it impossible to detect SSC in brain extracts.

3.3. Ion-modified reversed-phase LC (IM-RPLC)

In IM-RPLC [25], an ion-modifying agent, which has an opposite charge to the ionic solutes to be separated, is added to the mobile phases. As mentioned before, SSC is an anion around the optimum detection pH, so the positively-charged reagents, tetrabutylammonium (TBA) perchlorate and cetyltrimethylammonium (CTMA) *p*-toluene sulfonate were used in this study. Although most commercially available ammonium salts are in the form of halides, sulfate and phosphate, they should be avoided in this detection system owing to their reactivity with mercury.

Even if 5 m*M* TBA is added to citrate buffer, the capacity factor k' of SSC is almost zero (data not shown). In contrast, the retention time of SSC is much longer when CTMA is used as an ion-modifying agent. The chromatograms in citrate, malonate and acetate buffers (pH 5.2) are shown in Fig. 4. It is clear from Fig. 4 that the k' of SSC is over 2 in all three different mobile phases. Further, GSSG, Cl^- and SSC show very different behaviors in the different buffers. Fig. 4a shows that Cl^- and SSC are not separated though they are both well separated from GSSG in citrate buffer. In malonate buffer with other conditions unchanged, SSC and Cl^- are separated, but GSSG almost coelutes with SSC and shows a tailing and very asymmetrical peak (Fig. 4b). While acetate buffer was found to inactivate the electrode at very low flow-rates (less than 0.1 ml/min) and make the SSC peak severely tailing in anion-exchange HPLC, it separated the Cl^-, SSC and GSSG effectively here, which is clearly shown in Fig. 4c. It is evident from Fig. 4c that the sensitivity to SSC is also good.

As seen from Fig. 4a–c, the retention times of Cl^-, SSC and GSSG on this column are quite sensitive to the nature of buffer. The values of k'_{Cl-}, k'_{SSC} and k'_{GSSG} are 2.2, 2.0 and 0.86, respectively, in citrate buffer, compared to the corresponding values of 4.3, 3.3 and 3.1 in malonate buffer. In contrast, the corresponding capacity factors of Cl^-, SSC and GSSG are equal to 13.4, 9.0 and 36.4 in acetate buffer. The capacity factors of Cl^-, SSC and GSSG increase as the buffer is changed from citrate to malonate and finally to acetate. This result is as expected from the ionic strengths of the buffers. The average negative charges on citrate, malonate and acetate are respectively estimated to be 1.8, 1.2 and 0.7 at the ambient pH (5.2). This declining trend leads to a decrease in the ionic strength of the corresponding buffer.

SSC and Cl^- have one net charge, while GSSG has two. The retention of GSSG is thus more strongly dependent on ionic strength than that of the monoanions. Of the monoanions, SSC is the larger so the electrostatic interaction between SSC and the cation-modified column stationary phase is thus expected to be weaker than that between Cl^- and the stationary phase. This expectation may explain the fact that the capacity factors of SSC are slightly smaller than those of Cl^-. However, it seems difficult to give a reasonable explanation for the behavior of GSSG in these buffers based only on electrostatics.

Based on the capacity factors, the best chromatographic conditions were obtained in acetate buffer. A linear relation (Fig. 5) between peak area and SSC concentration was observed from 0.050 to 10 μ*M* and the detection limit was about 8 n*M* or 0.16 pmol under these separation conditions. A chromatogram from a mixture of 20 μ*M* cysteine (CySH), cystine (CSSC), glutathione (GSH), oxidized glutathione (GSSG), NaCl and *S*-sulfocysteine (SSC) is given in Fig. 6. It is seen from Fig. 6 that SSC is also well separated from other related species in biological samples.

This satisfactory performance has been further observed in brain extract. Fig. 7 is a chromatogram of an acetonitrile extract of neonatal rat brain spiked with 1.25 μ*M* SSC. As compared to Fig. 2, Fig. 7 exhibits both better separation and higher sensitivity for SSC. Whether cysteine administration to the rat will result in an increase in SSC concentrations in rat brain will be investigated in the future.

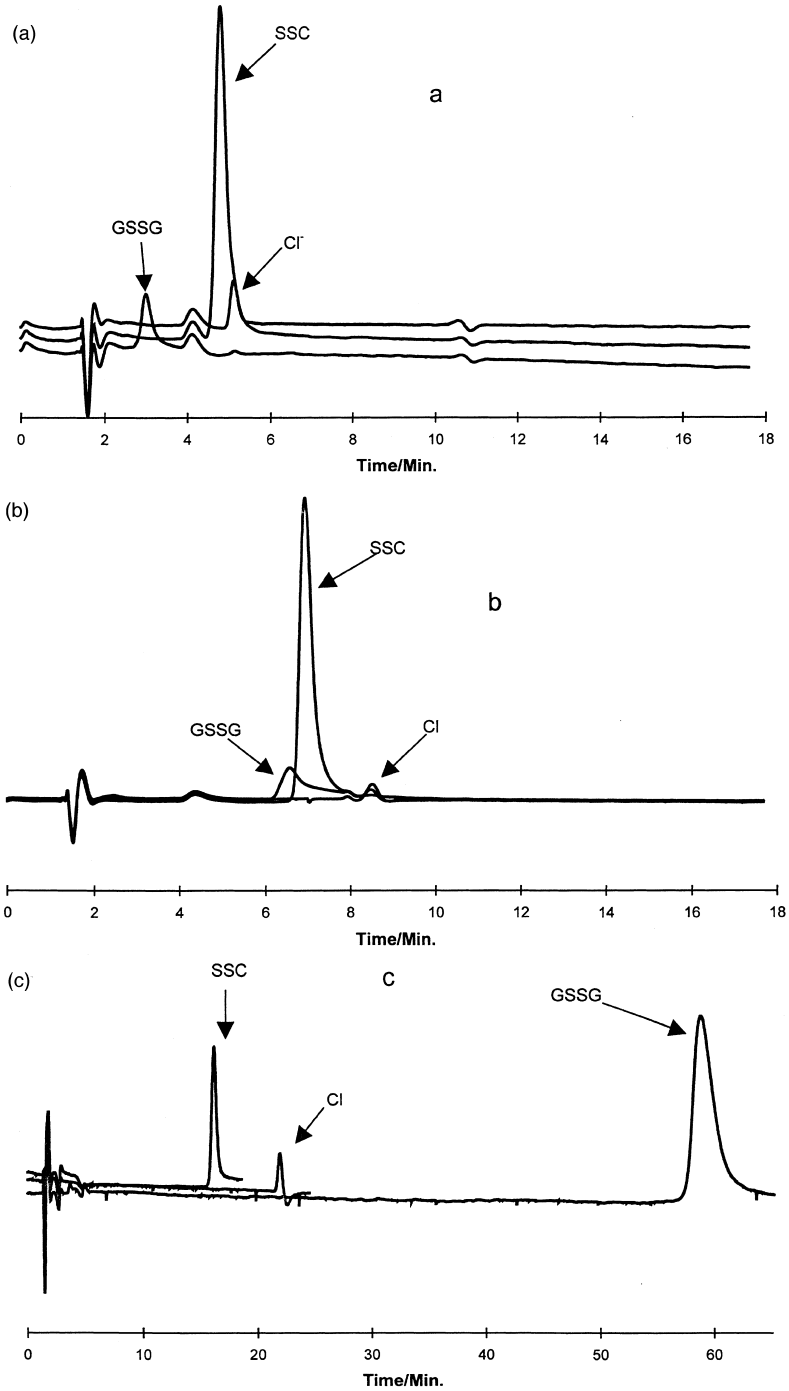

Fig. 4. Separations of GSSG, Cl^- and SSC by ion-modified reversed-phase liquid chromatography in (a) 10 m*M* citrate, (b) 10 m*M* malonate and (c) 20 m*M* acetate. Column: Zorbax XDB-C8, 5 μm, 150×2.1 mm. Ion-modifying reagent: 0.5 m*M* cetyltrimethylammonium *p*-toluenesulfonate (CTMA). With only one exception in case c, where the concentration of SSC was 5 μ*M*, 20 μl of 50 μ*M* standards were injected.

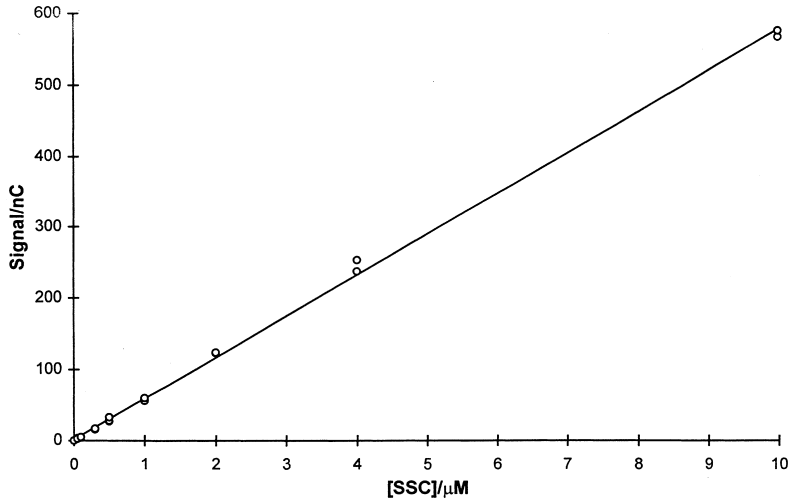

Fig. 5. Plot of peak area vs. concentration of SSC from zero to 10 μ*M*. Each concentration is represented by two or three 20-μl injections.

4. Conclusions

SSC detection was limited by the interference peak from Cl^- in rat brain tissue samples. The response of Cl^- in our detector can be decreased but not eliminated by decreasing the potential at the downstream electrode. A silver cartridge can remove Cl^- from micromoles to several millimoles, but in application to real samples it was found to introduce an interfering peak. A good compromise between separation and detection was not possible in anion-exchange chromatography. The samples are successfully separated by ion-modified reversed-phase LC in acetate buffer at optimum detection pH 5.2. Most

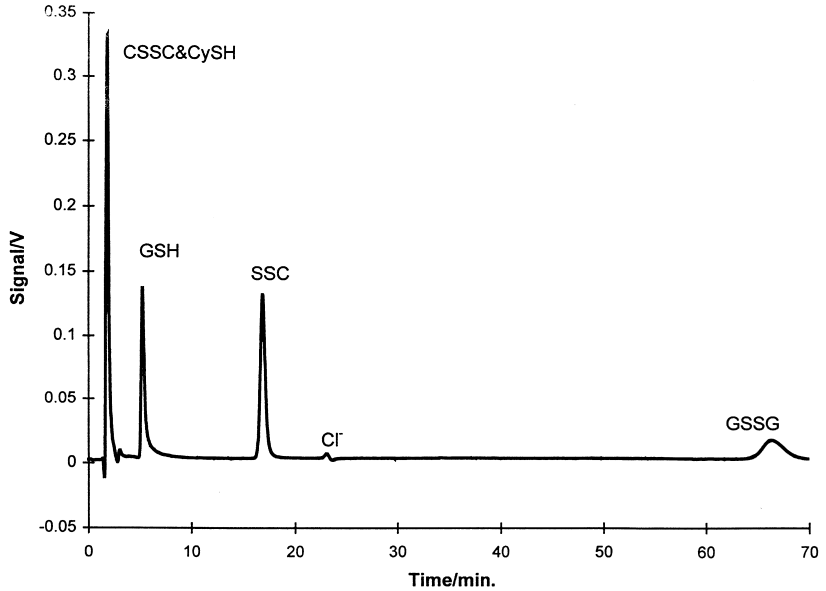

Fig. 6. Chromatogram of a standard mixture of 20 μ*M* CySH, CSSC, GSH, GSSG, NaCl and SSC.

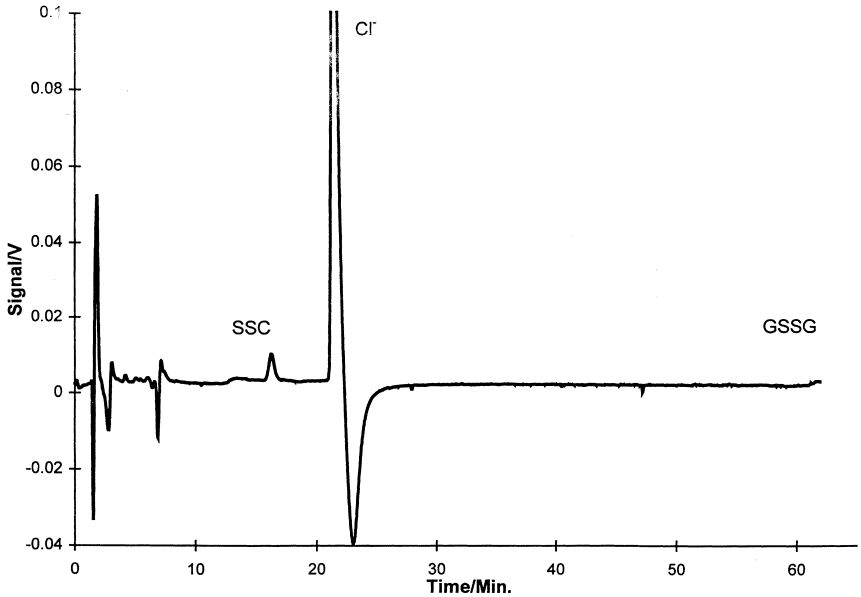

Fig. 7. Chromatogram of a rat brain extract spiked with 1.25 μ*M* SSC. Acetonitrile (100%) was used as the extraction medium.

importantly, a detection limit of 8 n*M* was achieved at the same time. In addition, SSC has been well separated from other common biological thiols and disulfides (e.g. CySH, GSH, CSSC), which are also active for this detector.

Acknowledgements

This project was supported by the NIH through Grant GM 44842.

References

[1] J.W. Olney, C.H. Misra, T. deGubareff, J. Neuropathol. Exp. Neurol. 34 (1975) 167.
[2] J.T. Coyle, P. Puttfarcken, Science 262 (1993) 689.
[3] B.K. Siesjo, K. Katsura, Q. Zhao, J. Folbergrova, K. Pahlmark, P. Siesjo, M.L. Smith, J. Neurotrauma 12 (1995) 943.
[4] D.K. Patneau, M.L. Mayer, J. Neurosci. 10 (1990) 2385.
[5] W. Moore, H.L. Wiener, A. Meister, J. Biol. Chem. 262 (1987) 16771.
[6] J. Martensson, A. Jain, E. Stole, W. Frayer, P.A. Auld, A. Meister, Proc. Natl. Acad. Sci. USA 88 (1991) 9360.
[7] G. Selema, R.M. Cristofol, S. Gasso, R. Griffiths, E. Rodriguez-Farre, J. Neurochem. 68 (1997) 1534.
[8] F. Irreverre, S.H. Mudd, W.D. Heizer, L. Laster, Biochem. Med. 1 (1967) 187.
[9] G.K. Brown, R.D. Scholem, H.B. Croll, J.E. Wraith, J. McGill, J. Neurol. 39 (1989) 252.
[10] G.L. Arnold, C.L. Greene, J.P. Stout, S.I. Goodman, J. Pediatr. 123 (1993) 595.
[11] J.W. Olney, O.L. Ho, Nature 227 (1970) 609.
[12] A. Lehmann, H. Hagberg, O. Orwar, M. Sandberg, Eur. J. Neurosci. 5 (1993) 1398.
[13] A. Slivka, G. Cohen, Brain Res. 608 (1993) 33.
[14] M. Sandberg, X. Li, S. Folestad, S.G. Weber, O. Orwar, Anal. Biochem. 217 (1994) 48.
[15] W.L. Xia, S.J. Woltman, M. Sandberg, S.G. Weber, J. Electroanal. Chem., in press.
[16] S.M. Lunte, P.T. Kissinger, J. Liq. Chromatogr. 8(4) (1985) 691.
[17] T. Kuninori, J. Nishiyama, Anal. Biochem. 197 (1991) 19.
[18] L.A. Allison, R.E. Shoup, Anal. Chem. 55 (1983) 8.
[19] R.F. Bergstrom, D.E. Kay, J.G. Wagner, J. Chromatogr. 222 (1981) 445.
[20] R. Eggli, R. Asper, Anal. Chim. Acta 101 (1978) 253.
[21] F. Kreurig, J. Frank, J. Chromatogr. 218 (1981) 615.
[22] L.A. Allison, J. Keddington, R.E. Shoup, J. Liq. Chromatogr. 6 (1983) 1785.
[23] C.F. Poole, S.A. Schuette, Contemporary Practice of Chromatography, Elsevier, New York, 1984.
[24] M.K. Radjai, R.T. Hatch, J. Chromatogr. 196 (1990) 319.
[25] J. G Chen, S.G. Weber, L.L. Glavina, F.F. Cantwell, J. Chromatogr. A 656 (1993) 549.