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Development of a liquid chromatographic method for picomole determination of *S*-sulfocysteine in trifluoroacetic acid extracts of neonatal rat brain

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Abstract

Neonatal Sprague–Dawley rat brain tissue was extracted with methanol, acetonitrile, acetic acid and trifluoroacetic acids (TFA). Among the extractants tested, 0.1 M TFA gave the highest recovery, $73.4 \pm 5.2\%$ (slope of regression of 'added' vs. 'found' and standard error of the slope) of *S*-sulfocysteine (SSC). The poorest recovery of SSC was found with acetonitrile and 90% methanol extractions (less than 10%). Possible reasons for the low recoveries have been explored. The recovery of SSC from aqueous standards in 0.1 M TFA is $92 \pm 5\%$. Detection of picomole quantities of SSC has been demonstrated with a combination of the optimized extraction procedures and our previously developed detection system. Supernatant of rat brain homogenate (0.10 M TFA as extractant) was evaporated to dryness in a vacuum centrifuge. Residues were reconstituted with deionized water. Samples were separated on a reversed phase column. The mobile phase was 20 mM aqueous acetate buffer (pH 5.2) containing 0.40 mM cetyl trimethylammonium *p*-toluene sulfonate and 2 vol.% methanol. Electrochemical detection used dual series gold-mercury amalgam electrodes. For the first time, *S*-sulfocysteine was detected in normal neonatal rat brain. Its concentration is 0.99 ± 0.25 pmol/mg brain tissue. The results indicate that TFA, rarely reported an an extractant, efficiently recovers SSC from rat brain tissues.

Keywords: Anoxia; Electrochemical detection; Excitotoxicity; Neurotoxicity; NMDA receptor

1. Introduction

S-sulfocysteine (SSC) is an effective agonist for the *N*-methyl-D-aspartate (NMDA)-receptor and was found to be excitotoxic over 2 decades ago [1–3]. SSC is suspected to be involved in some neuropathological disorders, e.g. sulfite oxidase deficiency which may lead to severe neurological dysfunction, dislocation of the lenses and excess accumulation of SSC in urine and plasma [4,5]. Molybdenum cofactor deficiency is another example resulting in neonatal seizures and neurological abnormalities in which a high concentration of

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SSC was found in urine [6]. *Salmomella typhimurium* has been observed to assimilate thiosulfate through SSC [7,8]. It is reported that SSC may participate in biosynthesis of cysteine in certain fungi [9].

Cysteine is an amino acid that is neurotoxic when injected systemically in neonatal rats [10]. Increased cysteine in brain is found after ischemia and cysteine may thus be involved in the neurodegeneration following stroke [11]. Interestingly, high cysteine concentrations have been reported in plasma of patients suffering from Alzheimer's and Parkinson's diseases and cysteine has been implicated as one possible toxin in AIDS dementia [12,13]. Although the neurotoxicty of cysteine is blocked by NMDA-receptor antagonists [14] the mechansim(s) and the NMDA-receptor ligand(s) involved are not fully clear as cysteine per se is a weak NMDA-receptor agonist [15]. Several hypothesis to explain the toxicity of cysteine have been put forward. One possibilty is that cysteine carbamate, formed from carbon dioxide and cysteine, could mediate the toxicty [14]. Other putative toxic metabolites include cysteine sulfinate. We have earlier shown that the concentration of CSA is moderately increased after cysteine injections in neonatal rats [16]. The conclusion was that CSA is not contributing to cysteine toxicity but that metabolism of cysteine to cysteine sulfinate is increased. In another study we have demonstrated that cysteine (or some cysteine metabolite) increases the extracellular levels of glutamate which thus could act as a toxic mediator [17]. Yet another possibility is that SSC, a very potent NMDA-receptor agonist [18], could be formed via transamination of cysteine sulfinate metabolism to β-sulfinyl pyruvate followed by spontaneous breakdown to sulfite and pyruvate [19]. Sulfite in turn could react with cysteine to form SSC. Elevated SSC could be neurotoxic also by inhibition of the rate limiting enzyme in synthesis of glutathione [20], thereby reducing the intracellular levels of this important free radical scavanger. As an initial step in the study of the role of SSC in normal and pathological brain we have here developed a method for the determination of endogenous SSC in neonatal rat brain.

There are no reports, to our knowledge, about the existence of SSC in rat brain, possibly due to the lack of an appropriate extraction procedure and a sensitive detection method. Based on the Au–Hg dual electrode system, initially designed to detect thiols and disulfides [21–23], an electrochemical detection system coupled to HPLC has been developed for the detection of SSC [24]. The detection is accomplished by SSC reduction with S–S bond cleavage at the upstream electrode and a complex reaction of mercury with the produced species at the downstream electrode. Recently, ion-modified reversed-phase liquid chromatographic separation conditions have been optimized for the electrochemical detector [25]. A detection limit of 8 nM (or 0.16 pmol) has been achieved for SSC detection in aqueous samples. The anticipated low concentration of SSC in brain necessitates the development of an appropriate sample preparation procedure compatible with the detection system.

For most solid tissues, typical extraction procedures include immediate homogenization following removal of the tissue or rapid freezing with later homogenization. Usually, a strongly acidic medium is employed to inhibit and denature enzymes and other proteins. A wide variety of acids such as perchloric, trichloroacetic, metaphosphoric, hydrochloric and acetic acids [26–34] have been used. As protein denaturants, organic solvents such as methanol, ethanol, acetone and acetonitrile have proven to be useful in some special cases [28,32–35]. In the present case, sample preparation must be compatible with the liquid chromatographic separation and electrochemical detection. Species such as Cl^-, SO_4^{2-} and PO_4^{3-} can interfere with the detection of SSC and should not be introduced during sample preparation.

Several acids including TFA and organic solvents have been tested as extraction media in the present study. Among these, TFA, commonly used in modern reversed-phase HPLC of amino acids and peptides but rarely reported as an extraction medium, has been found to give the highest recovery of SSC from the spiked rat brain tissues.

Using the optimized sample preparation procedures, neonatal brain tissue extracts have been analyzed based on the previously described detection system [24,25]. A low concentration of SSC, 0.74 pmol/mg brain tissue has been found in normal neonatal rat brain.

2. Materials and methods

2.1. Chemicals

L-*S*-sulfocysteine, glutathione (GSH) and cetyltrimethylammonium *p*-toluenesulfonate (CTMA) were purchased from Sigma (St. Louis, M)). Other chemicals like methanol, TFA, acetic acid (AcH) and acetonitrile (CH_3CN) were analytical reagent grade or better and obtained from commercial sources. Water was doubly deionized with a Milli-Q system (Millipore, Bedford, MA). Triple-distilled mercury was from the Bethlehem Apparatus (Hellertown, PA).

2.2. Chromatography and detection system

A syringe pump (ISCO, Lincoln NE, Model 100 DM) was used in conjunction with a dual Au-Hg amalgam electrode detection system, in which a potentiostat (LC-4B (BASW. Lafayette, IN)) and a thin layer cell (BAS) were employed. A Rheodyne HPLC injector with a 20 μl loop was used for all injections. The mobile phase was 20 mM aqueous AcH solution containing 0.4 mM CTMA and 2% methanol adjusted to pH 5.2 with aqueous NaOH solution. The mobile phase was continuously degassed by purging nitrogen through the reservoir. All separations used a C18 column, 150 × 2.0 mm, 5 mm, 300 Å pore size (Phenomenex, Torrance, CA) at a flow rate of 0.2 ml/min at room temperature. EZChrom (Scientific Software) was installed in a Micron P-75 PC to collect and analyze the data.

The 3 mm diameter Au–Hg dual amalgam electrodes were prepared as described previously [24,25]. The potentials applied to the upstream and downstream electrodes were −1.5 V and +0.15 V vs. Ag/AgCl, respectively. Only the downstream electrode was monitored in this study. Two 0.0125 cm thick spacers were used to give a channel height of 0.025 cm.

2.3. Preparation of Sprague–Dawley rat brain extracts

All the brain extracts used in the current study were prepared in the Gothenburg, Sweden laboratory. Immediately after decapitation of the neonatal rats (4 days), the heads were frozen in liquid N_2 and stored at −80°C. Prior to extractions, pieces of cerebral cortex were dissected out in a cryostat held at −15°C or on dry ice. The weighed and frozen tissue was homogenized (10 strokes) in an extraction medium (10% wt/vol.) in which SSC was added at different concentrations for tests of recovery. The extraction media used were water, acetonitrile, 90% (v/v) methanol, 0.1 M AcH and 0.1 M TFA. To remove tissue residues and protein, the homogenates were centrifuged at 13 000 × *g* for 30 min at +4°C. The supernatants of the tissue homogenates were evaporated to dryness in a Speedvac (Model RH 40-12, Savant Instruments, Hicksville, NY) overnight in a cold room. Each tube contained 200 ml of supermutant, i.e. about 20 mg tissue. Minor modifications to the above procedures can be found in Section 3.

The dried extracts were shipped to Pittsburgh, and were usually received within 1 week. Each tube was reconstituted with 200 μl water. The solutions were ultrasonicated for 5 min followed by 10 min centrifugation. The samples were then filtered through a 0.45 μm Nylon Acrodisc 13 filter (Gelman Sciences, Ann Arbor, MI) before injection.

2.4. Standard preparation

Standards of SSC were prepared both in Sweden and in Pittsburgh. In the former case, freshly made high concentration SSC solutions were diluted to the desired concentration, 200 μl of which were evaporated and treated as above. Standards of SSC and GSH were prepared in Pittsburgh in doubly deionized water as described elsewhere [24,25].

3. Results and discussion

3.1. Stability of SSC standards

Since it usually took 3 days to 1 week to receive the samples from Sweden, the stability of SSC during manipulations was initially examined in different extraction media. Four different concentrations of SSC were prepared in water, acetonitrile, 90% methanol, 0.1 M AcH and TFA, respectively, in Sweden. A total of 200 μl of these SSC standards were treated as brain extracts. These are considered the 'samples'. The dried samples were reconstituted in Pittsburgh and compared with a set of freshly made SSC standards, considered as 'controls'. The recovery of SSC was calculated by comparing the slope of a calibration curve based on the shipped standards to that from the controls. The results in Table 1 show an efficient recovery of SSC in all five extractants. The error in the ratio of the slopes is a function of the standard errors of the two slopes. The error in the slope of the controls is negligible compared to the error in the sample slopes, so the stated error is just the error in the sample slopes. Thus, SSC is not significantly degraded during sample processing and transportation.

3.2. Recovery of SSC in various extraction media

In the concentration range from 0.5 to 4 μM (or higher), the recoveries from brain extracts of exogenously added SSC were determined under various extraction conditions (Table 1). Since the supernatants from the water extractions were opalescent, they were not tested. It was found that 0.1 M TFA resulted in the highest recovery of SSC. While the recovery of SSC in 0.1 M acetic acid was intermediate, it was poor in both acetonitrile and 90% methanol.

Table 1
The recoveries of SSC in different media in the presence and absence of brain extract

Solvent	Recovery* (extract absent) (%)	Recovery (extract present) (%)
Water	94.8 ± 2.0	–
90% Methanol	93.6 ± 2.9	4.7 ± 0.2**
Acetonitrile	92.5 ± 1.0	$-0.94.0$
0.1 M AcH	103.7 ± 0.5	42.3 ± 9.8
0.1 M TFA	98.7 ± 3.0	73.4 ± 5.2

* Recoveries were obtained from 3–4 points in the concentration range from 0.5–4 μM.

** The concentration range of SSC is from 0.1–10 μM.

3.3. Influences of ultrasonication and filtering

The poorest recovery of SSC was observed in acetonitrile (Table 1). To test the influence of sonication, 1 μM SSC samples with or without sonication were examined. Surprisingly, it was found that the non-sonicated samples gave a 15% higher recovery than those sonicated in acetonitrile. However, sonication has no significant effect ($< 5\%$) on SSC in a moderately acidic medium such as AcH. Therefore, sonication may be partially responsible for the poor recovery in acetonitrile, but its effect is negligible in acidic solutions. In the present study, therefore, sonication can be employed for fast extraction in acidic solution.

To evaluate the possible effects of filtering the SSC solutions prior to chromatography, freshly prepared SSC standards in water and mobile phase were compared with tissue samples with added SSC. The data in Fig. 1 indicate that filtering lowered the recovery by about 30% in water. Fortunately, no effect of filtering on SSC recovery was observed for the tissue samples. Probably the loss from filtering is due to the sorption to filter membrane by an ion-exchange process, since SSC is an anion over a wide range of pH. This hypothesis was supported by the fact that the filter effect is negligible (less than 2%) for SSC standards prepared in the mobile phase instead of pure water. The large ionic strength in the mobile phase or in tissue samples may have inhibited ion-exchange reactions between SSC and the filter.

3.4. Interactions between SSC and GSH or other species in brain extracts

It is known that reduced glutathione (GSH) is present in mM concentrations in rat brain. Both

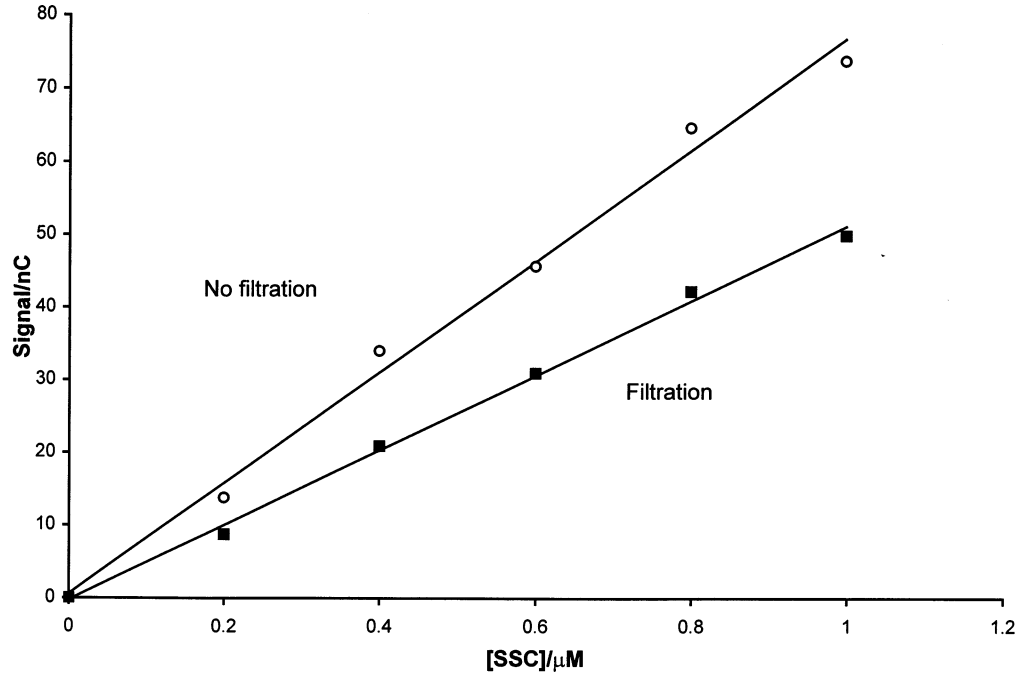

Fig. 1. The effect of filtration on SSC standards in water. The results from linear regression are $y = 76.2\ (3.4)x + 0.6\ (2.05)$ for the unfiltered samples and $y = 51.4\ (1.4)x - 0.3\ (0.85)$ for the filtered samples.

enzyme-dependent and enzyme-independent exchange reactions between GSH and thiosulfates have been demonstrated to be possible in vitro [32]. To test whether this kind of reaction could reduce the recovery of SSC, mixtures of GSH and SSC were made in water, 0.1 M acetic acid and 0.1 M TFA and kept at room temperature. The concentration of GSH was 50 mM, while the SSC concentration varies from 0 to 4 μM. After 3 days incubation at room temperature, SSC concentrations were determined. Results were compared to freshly made SSC standards. A straight line was obtained for each series of incubated solutions as well as the freshly made SSC standards. As compared to the slope of SSC control, the relative values of the slopes in water, 0.1 M AcH and 0.1 M TFA are $100.2 \pm 2.5\%$, $96.2 \pm 0.1\%$ and $99.1 \pm 2.0\%$, respectively. These results indicate that no significant interaction between GSH and SSC takes place even at room temperature for 3 days. Similarly, there is no reduction in SSC concentration when excess GSH is added to SSC spikes of brain extracts. The observations imply that enzyme-independent reactions between GSH and SSC are not the reason for less than complete recovery of SSC.

However, the above conclusion does not rule out the possible reactions between SSC and other species in the extracts that could facilitate the degradation of SSC before applying the prepared solution to column. Therefore, brain extracted with TFA containing 0.5 μM SSC were reconstituted and kept at room temperature for 72 h, were stored at -20°C for 72 h, or were injected immediately. SSC concentration decreased by 20% if the extract was stored at room temperature. However, SSC was stable in the frozen samples. These results imply that some interactions between SSC and other species can lead to the loss of SSC and thus long time delays, i.e. days, after sample reconstitution should be avoided.

During biological sample preparation, enzymatic reactions may occur in the homogenization and centrifugation steps. Table 1 indicates that enzymes may play an important role in the loss of SSC, since the best recoveries were observed in

TFA and AcH, which are protein precipitants. As a matter of fact, an enzyme-dependent interchange between thiosulfate ester and GSH has been demonstrated to occur [36]. While the possibility of enzyme-independent exchange between GSH and SSC has been ruled out here, it is possible that enzyme-dependent exchange may contribute to the loss of SSC.

3.5. SSC recovery and determination of SSC in rat brain tissue

Preliminary experiments demonstrated that the concentration of SSC in neonatal rat brain is below 0.5 μM. A series of experiments, only using TFA, were performed in the range of 100–500 nM. Aqueous and brain samples were treated according to the extraction procedure in the Gothenburg lab and mailed to the Pittsburgh lab. Duplicate injections were made of each sample, and several samples were prepared at each concentration. An efficient recovery, $92 \pm 5\%$, of SSC in 0.1 M TFA solution without tissue, was observed in this concentration range (Fig. 2a). This indicates that SSC at low concentrations is stable during sample manipulations in 0.1 M TFA. The recovery of SSC added to rat brain tissue was $75 \pm 6\%$ (Fig. 2b). The intercept is 0.074 ± 0.018 μM in Fig. 2b. The level of SSC in brain tissue was calculated from the intercept and the recovery to be 9.9 ± 2.5 nM (which is identical to the procedure of standard additions), which corresponds to 18.8 ± 5.0 pmole of SSC in 20 mg rat brain. Thus, the endogenous SSC concentration was estimated to be 0.99 ± 0.25 pmol/mg rat brain tissue in normal neonatal rat brain. Typical chromatograms of rat brain extracts are given in Fig. 3.

4. Conclusions

A greater than 90% recovery of SSC was demonstrated in H_2O, AcH, TFA, methanol and acetonitrile. In the presence of brain tissue, the highest recoveries of SSC, about 75% both in the range from 0.5 to 4 μM and also in the lower concentration range (below 0.5 μM), were found using 0.1M TFA as the extracting medium. For the first time, endogenous SSC was detected in normal rat brain. The concentration of SSC was estimated to be about 1 pmol/mg wet rat brain tissue via HPLC-EC analysis.

The present study indicates that organic solvents (acetonitrile and methanol) should be avoided even though they can be readily evaporated and are evidently compatible with the HPLC system. Before injection to an HPLC system, biological samples are usually filtered. The results presented in the current study indicate that filtering may have some negative effects on the recovery from aqueous solutions. While the mechanisms leading to low recovery of SSC in the extraction media such as methanol or acetonitrile are not completely understood, enzyme-dependent

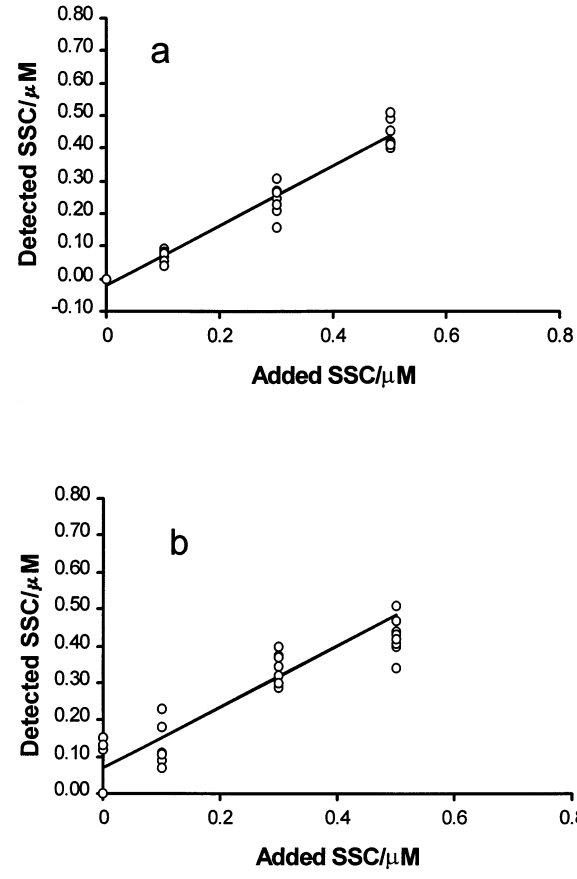

Fig. 2. The recoveries of *S*-sulfocysteine in the absence (a) or the presence of rat brain extract (b) in 0.1 M TFA.

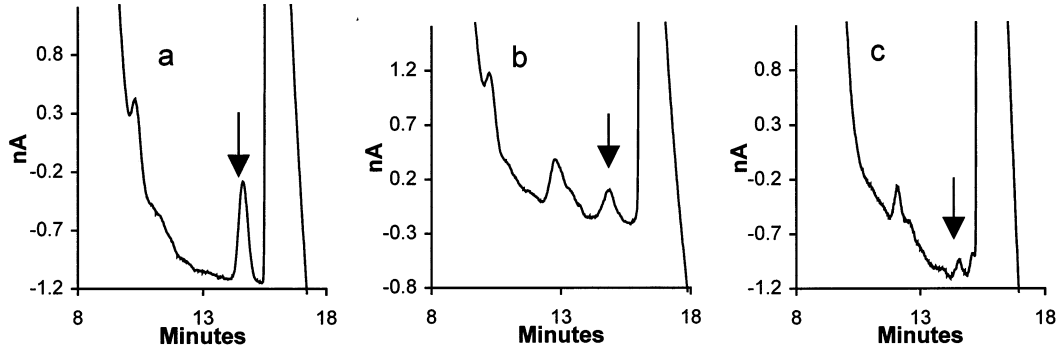

Fig. 3. A typical chromatogram of neonatal rat (4 days) brain sample extracted by 0.1 M TFA. The SSC peaks are shown by the arrows. (a) 500 nM spike in brain tissue (b) extract of neonatal rat brain 3 h after ip injection of cyteine (c) extract from neonatal rat brain with no treatment.

reactions may play an important role. This was partly supported by the observation that the recovery of SSC was higher in acidic media. TFA is rarely reported as an extraction medium for biological matrices. In the present study, TFA was found to be the most suitable extraction medium.

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