Journal of Chromatography B, 877 (2009) 3434–3441

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Measurement of sulfur-containing compounds involved in the metabolism and transport of cysteamine and cystamine. Regional differences in cerebral metabolism☆

John T. Pinto[a], Tetyana Khomenko[b,d], Sandor Szabo[b,d], Gordon D. McLaren[c,e], Travis T. Denton[f], Boris F. Krasnikov[a], Thomas M. Jeitner[g], Arthur J.L. Cooper[a,*]

[a] Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA
[b] Diagnostic, Molecular Medicine, VA Long Beach Healthcare System, Long Beach, CA, USA
[c] Medical Health Care Groups, VA Long Beach Healthcare System, Long Beach, CA, USA
[d] Departments of Pathology and Pharmacology, University of California, Irvine, CA 90822, USA
[e] Department of Medicine, University of California, Irvine, CA 90822, USA
[f] Department of Chemistry and Biochemistry, Eastern Washington University, Cheney, WA 99004, USA
[g] Applied Bench Research Core, Winthrop University Hospital, Mineola, NY 11501, USA

ARTICLE INFO

Article history:
Received 27 February 2009
Accepted 19 May 2009
Available online 28 May 2009

Keywords:
Cysteamine
Cystamine
Thialysine
S-(2-aminoethyl)-L-cysteine ketimine
S-(2-aminoethyl)-L-cysteine ketimine-decarboxylated dimer
CoulArray detection

ABSTRACT

An HPLC method with coulometric detection is presented for the quantitation of cysteamine, cystamine, thialysine, glutathione, glutathione disulfide and an oxidized metabolite of thialysine [*S*-(2-aminoethyl)-L-cysteine ketimine decarboxylated dimer (AECK-DD)]. The advantage of coulometric detection is that derivatization is unnecessary if the analyte is redox sensitive. The method was used to quantitate several sulfur-containing compounds in plasma and brain following gavage feeding of cysteamine to rats. Cysteamine, cystamine, thialysine and AECK-DD were detected in the brains of these animals. Interestingly, cysteamine treatment resulted in greatly elevated levels of cerebral methionine, despite the fact that cysteamine is not a precursor of methionine.

1. Introduction

Formally, cysteamine [$H_2NCH_2CH_2SH$; 2-mercaptoethylamine] is the decarboxylated analogue of cysteine. However, in mammals, cysteamine is not formed from cysteine directly by decarboxylation. Rather, it is obtained from the metabolism of pantetheine, a component of coenzyme A [1,2] by the action of pantetheinase. In addition to formation of cystamine, the oxidized (disulfide) form of cysteamine, the main route for metabolism of cysteamine is thought to involve oxidation to hypotaurine followed by oxidation to taurine via sequential reactions catalyzed by specific monooxygenases [3]. Several methods for the estimation of cysteamine, hypotaurine and taurine have been described (e.g., [4–6]). Levels reported in the literature for free cysteamine in mammalian tissues vary by orders of magnitude (see the discussion by Pinto et al. [7]). This discrepancy is probably due to inadequate methodology in many cases. The most reliable estimates are that the background levels of *free* cysteamine in rodent tissues (e.g., [7–9]) and human plasma [10] are very low and generally below the detection limits of even the most sensitive techniques (generally <0.1 to <2.0 nmol/g of tissue). (See Ref. [10] for a discussion of methods used for cysteamine determination.) Coloso et al. [3] reported levels of cysteamine in mouse liver, kidney and brain of 0.16 ± 0.01, 0.35 ± 0.14 and 0.11 ± 0.10 μmol/g of tissue, after reduction of tissue homogenates with dithiothreitol (DTT). Pitari et al. [2] reported levels of cysteamine in mouse kidney and liver of ~15 and 24 nmol/g of tissue, respectively, after reduction of perchloric acid-deproteinized homogenates with mercaptopropionic acid. It is likely that most of the cysteamine measured in the experiments reported by Coloso et al. [3] and Pitari et al. [2] was in mixed disulfide linkages with protein thiols. Duffel et al. [11] previously reported that cysteamine occurs in rat liver and kidney in the form of mixed disulfides with protein cysteinyl residues at concentrations of about ~18–20 nmol/g tissue. Nevertheless, free cysteamine can be detected in rat tissues after administra-

☆ This paper is part of the special issue "Analysis of Thiols", I. Dalle-Donne and R. Rossi (Guest Editors).

* Corresponding author. Tel.: +1 914 594 3330; fax: +1 914 594 4059.
E-mail address: Arthur_cooper@nymc.edu (A.J.L. Cooper).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.05.041

tion of pharmacological doses of cysteamine. Thus, Ogony et al. [9] reported ~22 nmol of free cysteamine/mg protein in brain 30 min after intraperitoneal injection of 300 mg of cysteamine per kg body weight into adult rats.

In addition to metabolism to hypotaurine and taurine, some investigators have suggested that cysteamine (presumably released *in vivo* by reduction of cysteamine-mixed disulfides) may be incorporated into thialysine [*S*-(2-aminoethyl)-L-cysteine] by the action of cystathionine β-synthase, especially in the brain. Very little cysteamine dioxygenase is present in the brain and cysteamine oxidation occurs predominantly in the liver [3]. Brain contains, however, appreciable amounts of cystathionine β-synthase [12], and cysteamine is an alternative substrate to homocysteine for this enzyme [1]. The catalytic product arising from the interaction of cysteamine with cystathionine β-lyase is thialysine. We have been unable to find any reports of baseline levels of thialysine in mammalian tissues. However, thialysine has been detected in the urine of normal human adults at a concentration of about ~12.7 μg/mg creatinine (~88 nmol/mg creatinine) [13]. This finding suggests that thialysine is a normal metabolite. Indeed, Cavallini and colleagues have detected several metabolites of thialysine in bovine brain, including *S*-(2-aminoethyl)-L-cysteine ketimine (AECK) [the cyclized form of the α-keto acid arising from transamination of thialysine by glutamine transaminase K (GTK)], 1,4-thiomorpholine-3-carboxylate (a reduced form of AECK) and a tri-cyclic compound resulting from spontaneous dimerization and decarboxylation of AECK and given the trivial name aminoethylcysteine ketimine decarboxylated dimer (AECK-DD) [14–21]. Although the origin of AECK-DD in brain is not known, at least a portion may arise via endogenously generated thialysine. Possible metabolic pathways leading to the formation of thialysine, AECK, 1,4-thiomorpholine-3-carboxylate and AECK-DD from cysteamine in brain are shown in Fig. 1.

Within the past several years, evidence has accumulated that cysteamine and cystamine may have important biological roles. Formation of endogenous cysteamine correlates with the presence of a membrane-bound vanin-1/pantetheinase, an enzyme associated with sexual development and, also, homing of pro-T lymphocytes to the thymus. Interestingly, however, vanin-1$^{-/-}$ mice grow and develop normally and are resistant to inflammation and other types of oxidative injury induced by whole-body gamma-irradiation or certain drugs. This protection correlates with reduction of apoptosis and inflammatory response factors and can be reversed by treating vanin-1 null animals with cystamine [22]. Vanin-1 null animals exhibit decreases in activity of selenium-independent glutathione peroxidase. The decrease in enzymatic activity correlates with changes in hepatic glutathione *S*-transferase (GST) isoenzymes including a significant decline in GSTA3, an increase in GSTP1 and a marginal decrement in GSTM1. Cystamine administration to vanin-1$^{-/-}$ mice restores GSTA3 enzymatic activity without influencing protein expression, suggesting a function for cystamine in protein stabilization or folding [23]. In a mouse model for colitis, vanin-1 deficient mice are protected from trinitrobenzene sulfonic acid-induced colitis. Protection is reversible by cystamine that antagonizes the peroxisome proliferator-activated receptor (PPARγ). Through inhibition of PPARγ by cystamine release, vanin-1 permits production of inflammatory mediators by intestinal epithelial cells suggesting that vanin-1 may be a sensor of stress that exerts control over innate immune responses [24]. In rats with induced colitis, myenteric neurons exhibited increased transglutaminase-immunoreactivity and enzyme expression. In cultured myenteric neurons, incubation with retinoic acid (a transglutaminase inducer) exhibited increased neuronal apoptosis, whereas addition of cystamine significantly reduced the number of apoptotic neurons [25]. In a similar fashion, the vanin-1/cysteamine pathway may contribute to islet beta cell protection from streptozotocin-induced death. Administration of cystamine protected islets *in vitro* and compensated for vanin-1 deficiency *in vivo* [26]. Low levels of cystamine also protected SHSY5Y cells against dopamine-induced macroautophagy [27]. Thus, endogenous production of cysteamine through pantetheinase may have important cytoprotective and immune modulating function despite low concentrations.

Given the high glutathione (GSH)/glutathione disulfide (GSSG) ratio in most tissues, changes in endogenous production or administration of pharmacological doses of either cysteamine or cystamine alone will result in generation of both cystamine and cysteamine *in vivo,* thereby altering the endogenous ratio of this physiological thiol couple. Thus, understanding the ratio of cysteamine/cystamine relative to that of GSH/GSSG and other thiol/disulfide redox active mixed disulfides may be critical in defining mechanism of efficacy for therapeutic uses of either cysteamine or cystamine.

In addition to its general interest as a product of pantetheine and coenzyme A metabolism, cysteamine is of interest as an ulcerogen. Thus, gavage feeding of very high doses of cysteamine to rats reliably generates duodenal ulcers within 24 h [28–31]. In addition, cysteamine has been used for many years to successfully treat children with cystinosis (e.g., [32]). At least six studies have shown that administration of pharmacological doses of cystamine to Huntington disease (HD) mice results in prolonged life expectancy [27 and references cited therein]. Although not fully understood, Batten disease results in an accumulation of lipopigments that leads to neuronal death in the brain, retina and central nervous system. In clinical and preclinical studies, cysteamine delayed the development of neurological symptoms, reduced apoptosis and helped retain brain volume thus slowing disease progression [33].

Here we describe methods adapted from previous work [7] for the quantitation of underivatized, redox-active cysteamine, cystamine, thialysine, AECK-DD, GSH, GSSG, cysteine and methionine using HPLC with CoulArray detection. HPLC equipped with CoulArray detection provides a quick, convenient method for direct determination of redox-active metabolites and obviates the need for derivatization. Typical times for elution of metabolites plus re-equilibration of the column are about 12 min. The method was applied to the detection of some or all of the above-mentioned sulfur-containing compounds in plasma, red blood cells (RBC) and brain after gavage administration of pharmacological doses of cysteamine to rats. Although these experiments result in non-physiological levels of sulfur-containing compounds, they are useful nevertheless in defining possible pathways for either cysteamine or cystamine metabolism under normal physiological conditions and may offer clues to the biochemical mechanisms involved in the cytoprotection afforded by cystamine/cysteamine in Huntington disease, Batten disease, diabetes (islet cells) and other inflammation-related diseases. In summary, the present methodology should be of interest to those investigators studying metabolism of cysteamine and cystamine and other redox-active, sulfur-containing compounds in health and disease.

2. Materials and methods

2.1. Materials

Cysteamine-HCl, cystamine-2HCl, diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), L-cysteine-HCl, thialysine, GSH, GSSG, octane sulfonic acid, bovine serum albumin (BSA) and metaphosphoric acid (MPA) were obtained from Sigma Chemical Company (St. Louis, MO). The cysteamine-HCl used in animal experiments was obtained from Aldrich (Milwaukee, WI). OmniSolv^{R} solvents (acetonitrile and *N*,*N*-dimethylformamide) were HPLC grade and obtained

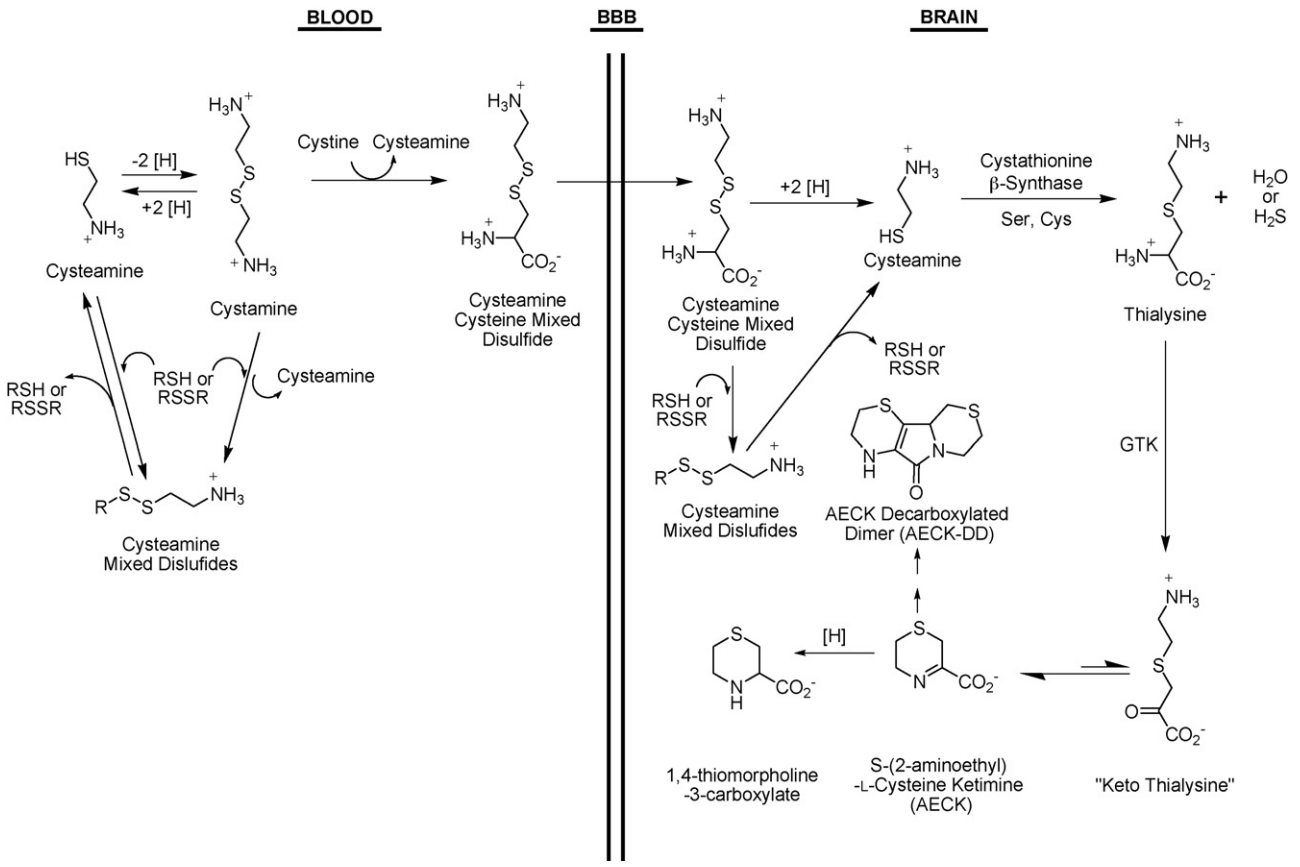

Fig. 1. Possible routes for entry of cysteamine into the brain and its metabolism to the potent anti-oxidant AECK-DD. Cysteamine–cysteine mixed disulfide is structurally similar to lysine and is predicted to cross the blood–brain barrier (BBB) on the lysine transporter. Once within the brain, the mixed disulfide is reduced to free cysteamine. A β-replacement reaction between serine (or cysteine) and cysteamine catalyzed by cystathionine β-synthase generates thialysine. Thialysine is a substrate of glutamine transaminase K (GTK). The α-keto analogue of thialysine generated by this reaction spontaneously cyclizes to a ketimine (AECK), which is either reduced in an enzyme-catalyzed reaction to 1,4-thiomorpholine-3-carboxylate or spontaneously decomposes to AECK decarboxylated dimer (AECK-DD). RSH can be cysteine, GSH or protein –SH. RSSR can be cystine, GSSG or disulfide linkages in proteins. The proposed metabolic pathway from thialysine to the heterocyclic compounds is based on work by Cavallini and colleagues [15–21].

from EM Science (Gibbstown, NJ). AECK-DD [systematic name 1,2–3,4–5,6–7,8-octahydro-1,8a-diaza-4,6-dithia-fluoren-9(8aH)-one] was synthesized according to the method of Antonucci et al. [34]. Its structure was confirmed by ^{1}H NMR and low resolution electrospray mass spectroscopy. Purity was assessed by melting point and gas chromatography–mass spectroscopy (GC–MS): mp (uncorrected) = 132–133 °C (Lit. 140–142 °C [28]); ^{1}H NMR ($CDCl_3$) δ 4.55–4.51 (m, 1H), 3.99–3.96 (m, 1H), 3.6–3.57 (m, 2H), 3.16–2.84 (m, 5H), 2.62–2.57 (m, 2H), 2.38–2.32 (m, 1H); LRMS (ESI) *m/z* calculated for $C_9H_{13}N_2OS_2$ $[M+H]^+$ 229.1, found 229.1; GC–MS >99% (t_R = 29.33 min, *m/z* 228 (100, M^+), 200 (35), 154 (45), 126 (15), 99 (9), 71 (9). The compound also yielded a single peak on HPLC analysis (see below).

The melting point was determined on a Fisher–Johns melting point instrument and NMR spectra were recorded at 400 MHz on a Varian unity spectrometer. Chemical shifts are reported in parts per million (ppm, δ) using the residual solvent peak (7.26 ppm in $CDCl_3$) as the internal standard. Low-resolution mass spectroscopy (LRMS) was carried out with a Micromass LCT time-of-flight mass spectrometer using electrospray ionization. Gas chromatography was performed on an Agilent Technologies 6890N Network GC System in split-mode with a ratio of 25:1 with helium as the carrier gas. The injection port temperature was held constant at 250 °C. The GC column used was 5% phenyl methyl siloxane (Zebron ZB-5, Phenomenex); capillary 30.0 m × 0.25 mm; film thickness 0.25 μm with a constant flow of He at 1.7 mL/min. The GC oven temperature, initially at 41 °C, was held at 41 °C for 3 min and then increased to 280 °C at 8 °C/min and held for 8 min. The detectors used were an Agilent 5973 network mass selective detector with the mass filter scanning from *m/z* 50 to *m/z* 800 under electron ionization conditions and a flame ionization detector held constant at 250 °C with hydrogen gas flow of 40 mL/min, air flow of 400 mL/min and the nitrogen makeup gas flow of 30 mL/min.

2.2. Animal experiments

The present study was approved by the Animal Study Subcommittee of the Veterans Affairs Medical Center in Long Beach, CA. The protocol used for cysteamine administration was that previously developed by members of our research group (TK, SS) to induce duodenal ulcers in male Sprague–Dawley rats [30,31]. This protocol was selected because animals tolerate gavage treatments of 250 mg/kg body weight of cysteamine-HCl, which permits metabolites of cyst(e)amine to be monitored within the vascular and central nervous system compartments. Rats (n = 15) received cysteamine-HCl (250 mg/kg body weight) using a protocol that involved three gavage treatments at 4-h intervals (0, 4, 8 h). Groups of rats were euthanized by CO_2 inhalation followed by cervical dislocation at 0, 2, 6, 12 and 24 h after administration of the first dose.

The brains were quickly removed and frozen in liquid nitrogen. Blood samples were removed by heart puncture and injected into BD Vacutainer™ plastic blood collection tubes (BD Diagnostics Preanalytical Systems, Franklin Lakes, NJ), containing EDTA as the anticoagulant, gently inverted 8–10 times and centrifuged at

1000 × g for 15 min in a fixed-angle rotor immediately after collection. The frozen brains, plasma and RBC were shipped on dry ice to the JTP/AJLC laboratory. While still frozen, the brain samples were cut with a scalpel into cerebrum and cerebellum. Note that EDTA generates a peak in the HPLC profile that interferes with the cysteine and cystine peaks. These amino acids therefore cannot be quantitated in EDTA-treated plasma.

2.3. Preparation of tissues for metabolite analysis

The procedure used for analysis of all the sulfur-containing compounds of interest, except AECK-DD, is a modification of that developed by Pinto et al. [7]. (A separate procedure was developed for AECK-DD; see below.) Five volumes of ice-cold 5% (w/v) MPA containing 5 mM DTPA were added to samples of frozen (−80 °C) rat tissues (50–75 mg) or plasma, which were then homogenized in a Dounce homogenizer. After incubation for 10 min on ice, the samples were centrifuged at 0 °C for 5 min at 13,000 × g in a microfuge to sediment coagulated protein. Precipitates were dissolved in 0.1 N NaOH and protein was quantitated by a spectrophotometric method using bicinchoninic acid reagent (Pierce Chemical Co., Rockford, IL). In many cases, supernatant fractions were analyzed immediately after removal of denatured protein for compounds of interest by using HPLC separation (see below). However, this was not possible in those experiments requiring consecutive analyses in tissue homogenates at time points separated by less than 12 min (i.e., the time for a complete HPLC run plus column re-equilibration). For these experiments, the tissue homogenate was deproteinized with 5% (w/v) MPA, stored briefly on ice, centrifuged, and then frozen at −80 °C until analysis could be completed. Control experiments showed that storage of processed samples in 5% (w/v) MPA at −80 °C for even 1 month has no effect on the recovery of cystamine, cysteamine, cysteamine metabolites (thialysine and AECK-DD) or GSH. Note that spiking of RBCs and tissue samples with GSH just prior to addition of MPA/DTPA denaturing agent does not result in measurable oxidation of the added GSH to GSSG (data not shown). Note also that it takes a few minutes for euthanization and removal of the brain, so that it is not possible to obtain a true zero time point for blood-derived cysteamine metabolites in brain. Zero time points in the experiments in which rats were gavage-fed cysteamine are referred to as "0" time points.

2.4. Determinations of redox-active sulfur-containing compounds by HPLC and coulometric detection

Concentrations of the redox-active sulfur-containing compounds of current interest were measured without prior derivatization by HPLC coupled with a coulometric detector [7]. The HPLC system consisted of an ESA-model 580 pump equipped with an 8-channel coulometric array (CoulArray) detector (ESA, Inc., Chelmsford, MA).

For the determination of all sulfur-containing compounds of interest, except AECK-DD, the supernatant fractions from the MPA homogenates were injected directly onto a Bio-Sil ODS-5S, 5-μm particle size, 4.0 mm × 250 mm, C18 column (Bio-Rad, Life Science Research Group, Hercules, CA) and eluted with a mobile phase consisting of 50 mM NaH_2PO_4, 0.05 mM octane sulfonic acid, 1% (v/v) acetonitrile and 0.5% *N*,*N* dimethylformamide (v/v) (pH 2.52) at a flow rate of 1 mL/min. All buffers following preparation are routinely degassed, filtered through a 0.2 μm Millipore nylon filter, and pH adjusted, if necessary. PEEK™ (polyetheretherketone) tubing was used throughout the HPLC system and a 0.2 μm PEEK™ filter was placed pre- and post-column to protect both column and flow cells, respectively, from any particulate matter. A Rheodyne injection valve with a 5-μL sample loop was used to manually introduce samples. The 8-channels of the CoulArray detector were set at 100, 200, 300, 400, 500, 600, 700 and 800 mV, respectively. Fig. 2A shows retention times (min) for a standard mixture of 1, metaphosphoric acid (1.6); 2, cysteine (2.2); 3, ascorbic acid (2.7); 4, cysteamine (3.9); 5, GSH (4.6); 6, uric acid (5.1) and 7, cystamine (6.6). Not shown in this elution pattern are methionine, which elutes at 9.6 min and oxidizes between 700 and 800 mV and thialysine, which elutes after 3.6 min and oxi-

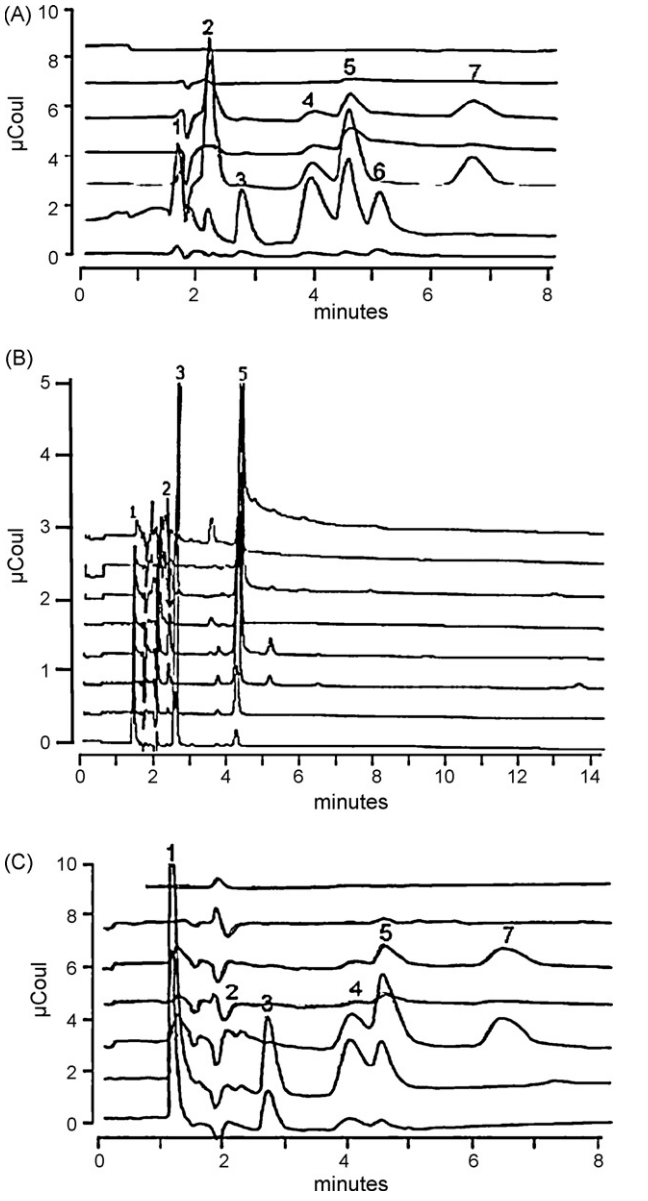

Fig. 2. HPLC elution profiles of redox-active compounds in a standard mixture and in a homogenate of rat cerebrum spiked with cyst(e)amine. (A) Retention times (min) for a standard mixture of redox-active compounds: (1) metaphosphoric acid (MPA) (1.6); (2) cysteine (2.2); (3) ascorbic acid (2.7); (4) cysteamine (3.9); (5) GSH (4.6); (6) uric acid (5.1); and (7) cystamine (6.6). The concentration of each compound except MPA was 20 nmol/mL. The 8-channel CoulArray detectors were set from bottom to top at 100, 200, 300, 400, 500, 600, 700, and 800 mV, respectively. The channel at 800 mV is not displayed on this figure as none of the compounds exhibited an oxidation potential above 700 mV. (B) Deproteinized homogenate of rat cerebrum showing endogenous levels of prominent redox-active compounds. Peaks 1, 2 (see arrow), 3 and 5 represent MPA, endogenous cysteine, ascorbic acid, and GSH, respectively. The original 20 min chromatogram was truncated as shown since no redox responsive metabolite was detectable after 15 min. (C) Deproteinized homogenate of rat cerebrum spiked with 20 nmol/mL each of cysteamine (peak 4) and cystamine (peak 7). Peaks 2, 3 and 5 represent endogenous cysteine, ascorbic acid, and GSH, respectively.

dizes between 600 and 800 mV. Fig. 2B illustrates a deproteinized 1/5 homogenate of rat cerebrum showing endogenous levels of prominent identified redox-active compounds. Peaks 1, 2, 3 and 5 represent MPA, and endogenous cysteine, ascorbic acid and GSH, respectively. Unnumbered peaks have not been identified. The original 20 min chromatogram was truncated for the figure at 15 min as shown since no redox responsive metabolite was detectable after that retention time. Fig. 2C shows a sample of rat cerebrum spiked with 20 μM each of cysteamine (peak 4) and cystamine (peak 7). Peaks 2, 3 and 5 represent endogenous concentrations of cysteine, ascorbic acid and GSH, respectively. Although mixed disulfides were not measured in the present study, the method can readily detect mixed disulfides. After preparation of known combinations of mixed disulfides, samples can be recognized by both their retention times and higher oxidation potentials relative to mercaptans.

To measure AECK-DD in brain the frozen tissue (1/5 w/v) was homogenized in 75 mM sodium citrate and 25 mM ammonium acetate, pH 7.5. After homogenization, a portion (0.5 mL) was removed and extracted 3 times with 0.5 mL of water-saturated $CHCl_3$ as described in [18]. Following each extraction and centrifugation at 2000 × *g* for 10 min, the lower chloroform layer was removed and placed into a clean Eppendorf tube. The combined extracts were placed in a vacuum rotavap and brought to dryness. Samples were reconstituted with 150 μL of 75 mM sodium citrate/25 mM ammonium acetate containing 50% acetonitrile, pH 7.84, vortexed and centrifuged at 2000 × *g* for 10 min. The supernatant fractions were injected onto an MD-150 column (3.0 mm × 150 mm; 3 μm particle size; ESA, Inc., Chelmsford, MA) and eluted at ambient temperature with a mobile phase consisting of 75 mM sodium citrate/25 mM ammonium acetate and 26% acetonitrile (v/v) (pH 7.84) at a flow rate of 0.8 mL/min. A Rheodyne injection valve with a 5 μL sample loop was used to manually introduce samples. The 8-channel CoulArray detectors were set at 100, 175, 225, 250, 275, 325, 400 and 500 mV, respectively. AECK-DD eluted at 4.28 min and exhibited maximum oxidation at 225 mV.

To assure standardization between analyses, calibration standards were interspersed at intervals among sample runs. Peak areas were analyzed using ESA, Inc. software. Recovery of authentic AECK-DD in a spiked brain homogenate was consistently about 97%. Concentrations of each metabolite were obtained from appropriate standard curves and are reported as pmol (or nmol)/mg protein. The range of linearity for the AECK-DD standard curve in our studies was from 0.5 to 750 nmol/mL and had an R^2 value of 0.997.

Data are reported as mean ± SEM.

3. Results and discussion

3.1. Mechanisms involved in cysteamine-induced duodenal ulcers

Previous studies from our laboratory demonstrated that cysteamine treatment reduces somatostatin bioavailability and markedly elevates serum gastrin levels, with an associated increase in gastric acid secretion [35–37], significantly decreased neutralization of acid in the proximal duodenum [38], decreased dopamine levels in glandular stomach and duodenum [39] and inhibition of gastric emptying and motility [40]. It is also known that certain transcription factors (e.g., Egr-1, HIF-1α) and their target genes play a key role in the pathogenesis of cysteamine-induced duodenal ulcers [30,31]. However, the complete mechanism of cysteamine-induced duodenal ulceration is still not clear. The present work should be of interest to those studying the biochemical mechanisms involved in the ulcerogenic action of cysteamine.

Table 1
Concentration of cysteamine and cystamine in red blood cells (RBC), plasma, cerebrum and cerebellum at various times following gavage feeding of cysteamine to rats.

Hrs	RBC	Plasma	Cerebrum	Cerebellum
Cysteamine (pmol/mg protein)				
"0"	0.2 ± 0.1	10 ± 8	91 ± 65	20 ± 14
2	46 ± 29	26 ± 13	816 ± 591	461 ± 317
6	217 ± 90	15 ± 9	3910 ± 1350	2000 ± 299
12	278 ± 66	266 ± 88	5210 ± 588	2450 ± 290
24	71 ± 40	45 ± 30	962 ± 546	840 ± 503
Cystamine (pmol/mg protein)				
"0"	NMA	3 ± 1	NMA	104 ± 4
2	0.2 ± 0.2	4 ± 3	NMA	163 ± 25
6	4 ± 1	115 ± 84	NMA	92 ± 31
12	6 ± 2	910 ± 260	NMA	134 ± 2
24	0.3 ± 0.2	91 ± 79	NMA	250 ± 8

NMA, no measurable amount (<0.2 nmol/mg of protein). $N = 3$ at each time point.

3.2. Cyst(e)amine, GSH and GSSG in plasma and RBC in rats gavage-fed cysteamine

At "0" time, the levels of both cysteamine and cystamine ranged from undetectable to low (<0.2 to ~10 pmol/mg protein) in plasma and RBC (Table 1). However, considerable free cystamine and cysteamine were detected in plasma following gavage feeding. The last of the three gavage feedings was at 8 h, and both cysteamine and cystamine peaked at about 12 h in the plasma. Cysteamine could be detected in RBC of the treated animals. Curiously, however, cystamine could not be detected in RBC (<0.2 nmol/100 mg protein) at any time point. This may be associated with high endogenous levels of GSH coupled with activity of glutathione reductase within RBC. Further support for this finding is shown in Table 2. At 6 h there was a slight decline in GSH and a concomitant increase in the levels of GSSG, suggesting that cystamine transported into RBC was reduced to cysteamine. The inability to detect cystamine was experimentally verified by spiking portions of RBC with cystamine immediately before addition of MPA. Recovery of cystamine was quantitative (data not shown). By 24 h, the level of cysteamine in plasma and RBC had greatly declined. The level of cystamine was also greatly diminished in the plasma by 24 h (Table 1).

Interestingly, considerable amounts of cystamine were found in the plasma. As with cysteamine, the cystamine is cleared rapidly from the plasma after the last gavage. Since cysteamine is the reduced form of cystamine, the high peak level of the oxidized form (i.e., cystamine) in the plasma was initially surprising. Since albumin contains seventeen disulfide linkages and one free sulfhydryl (Cys-34), it is possible that cystamine may have arisen from sulfide-

Table 2
Concentration of GSH and GSSG in red blood cells (RBC), plasma, cerebrum and cerebellum at various times following gavage feeding of cysteamine to rats.

Hrs	RBC	Plasma	Cerebrum	Cerebellum
GSH (nmol/mg protein)				
"0"	4.03 ± 0.29	0.006 ± 0.001	14.5 ± 1.0	7.31 ± 0.38
2	4.06 ± 0.24	0.022 ± 0.015	14.2 ± 2.1	11.0 ± 0.8
6	3.80 ± 0.92	0.038 ± 0.017	18.1 ± 1.0	10.0 ± 2.1
12	3.72 ± 0.38	0.038 ± 0.006	17.7 ± 0.7	7.18 ± 0.29
24	3.73 ± 0.16	0.046 ± 0.019	16.2 ± 2.3	11.5 ± 0.7
GSSG (pmol/mg protein)				
"0"	325 ± 101	81 ± 9	NMA	NMA
2	227 ± 12	150 ± 60	NMA	NMA
6	375 ± 32	212 ± 59	NMA	NMA
12	408 ± 64	588 ± 62	NMA	NMA
24	570 ± 165	294 ± 108	NMA	NMA

NMA, no measurable amount (<0.2 nmol/mg of protein). $N = 3$ at each time point.

disulfide interchange reactions with serum albumin. Although the majority of the disulfide bonds are protected from solvents and reducing agents, some are accessible to reduction. In this regard, we have found that bovine serum albumin (BSA) readily reacts with cysteamine. BSA was incubated with excess cysteamine in phosphate buffered saline (PBS) for 12 h at 37 °C. The BSA was precipitated with 5% MPA and washed several times with 5% MPA to remove unbound ligand. The pellet was then dissolved in 0.2 M KOH and reduced with potassium borohydride. Analysis of the supernatant fraction revealed the presence of cysteamine in a molar amount greater than that of the BSA, suggesting that at least one disulfide bond of BSA can participate in sulfide disulfide interchange reactions with cysteamine (unpublished data). It may also be relevant here to mention that blockade of the free sulfhydryl with cysteine, GSH, or even cysteamine can prevent albumin dimer formation [41]. Another explanation for the detection of cystamine in the plasma, but not in the RBC in the cysteamine-treated rats, involves the availability of redox metals in the two compartments. The oxidation of cysteamine can be stimulated by transition metals, such as iron and copper. *In vitro* studies have demonstrated that cysteamine reacts with transition metals (M^n, where *n* refers to the oxidation state). In the process, cysteamine is oxidized to its corresponding disulfide cystamine: $2RSH + 2\,M^n \leftrightarrows RSSR + 2\,M^{n-1} + 2H^+$ [42,43]. Cysteamine can generate H_2O_2 in the presence of fetal calf serum [43]. This suggests Fenton-type chemistry, but the origin of cysteamine-accessible Cu^{2+}/Fe^{3+} in plasma is not apparent.

3.3. Sulfur-containing brain metabolites in rats gavage-fed cysteamine

3.3.1. Cysteamine and cystamine in cerebrum and cerebellum

Table 1 shows the levels of cysteamine and cystamine at various time points over a 24-h period in cerebrum and cerebellum of rats gavage-fed cysteamine. The disposition of these two compounds is markedly different between cerebrum and cerebellum. Cysteamine at time "0" in both cerebrum and cerebellum was relatively low (<100 pmol/mg protein) but detectable. In both tissues, the level of free cysteamine increased in the cysteamine-treated animals one to two orders of magnitude by 12 h and declined considerably by 24 h. At 12 h, there was twice as much cysteamine in the cerebrum as in the cerebellum. By contrast, cystamine was below detectable limits in cerebrum over the entire 24-h period. However, a low level of cystamine was detected at time zero in the cerebellum and there was a trend toward increasing levels over the 24-h period. It is interesting, however, that despite massive increases of cysteamine in the brain, this is not reflected in a proportionate increase in cystamine, presumably reflecting the large GSH/GSSG ratio (>100:1) in rat brain [44]. If one assumes that 1 g wet weight of rat brain contains about 100 mg of protein and that the water content is 80%, then the peak levels of cysteamine in the cerebrum and cerebellum are about 0.6 mM and 0.3 mM, respectively.

In a previous study [7], we were unable to detect either cysteamine or cystamine (≤2 nmol/mg protein) in normal mouse brain after administration of cystamine in the drinking water (900 mg/L). In the present work, we detected cysteamine and cystamine in the rat cerebrum and cerebellum at time "0". We also detected cystamine in rat cerebellum at time "0". The apparent discrepancy may be due to species difference, but is most likely due to the significant time lapse between administration of cysteamine, euthanization and removal of the brain at the earliest time point. Thus, cysteamine and cystamine detected in the rat brain at "0" time is most likely a reflection of some uptake into the brain from the blood and some cysteamine and cystamine in the small blood compartment associated with the isolated brain.

3.3.2. GSH, cysteine and methionine in cerebrum and cerebellum

Because a key enzyme of the transsulfuration pathway (cystathionine γ-lyase) is of low activity in brain, it has generally been considered that the transsulfuration pathway is not important in neural tissues (e.g., [45]). However, it has recently been suggested that the brain possesses an intact transsulfuration pathway and that the pathway is linked not only to methionine homeostasis, but also to GSH homeostasis in the brain [46]. Thus, it was deemed important to assess the effect of cysteamine administration on key metabolites derived from the transsulfuration pathway in the brain, namely GSH (from cysteine), cysteine and methionine.

As was noted previously in mice chronically treated with cystamine [7,47], acute treatment of rats with cysteamine did not produce a significant change in brain GSH levels even by 24 h after the first dose of cysteamine (Table 2). This may be due to the very long turnover time for GSH in rat brain ($t_{1/2} \sim 3$ days) [48]. In previous studies, the level of cysteine in the whole brain was significantly increased in mice chronically treated with cystamine [7,47]. In the present work, cysteamine administration resulted in a 30% decrease of cysteine in the cerebrum, but an almost doubling of cysteine in the cerebellum (Table 3).

Possibly, competition by cysteamine for homocysteine at the active site of cystathionine β-synthase leads to lower levels of cystathionine and, consequently, to lower levels of cysteine in the cerebrum of the brains of the cysteamine-treated rats. On the other hand, the increased cysteine in the cerebellum may perhaps be due to increased uptake across the blood–brain barrier of the mixed disulfide of cysteine–cysteamine masquerading as a lysine mimic (Fig. 1). The brain contains an active uptake system for lysine and basic amino acids [49]. Once inside the brain, the mixed disulfide may be reduced to free cysteamine and cysteine.

Along with the decline of cysteine in the cerebrum, there was a marked increase of methionine from 200 pmol/mg of protein to approximately 900 pmol/mg of protein in the cysteamine-treated rats. There was also an increase of methionine in the cerebellum of the treated rats, but the increase was not as great (Table 3). The sulfhydryl of cysteamine is more electronegative than that of cysteine. Thus, cysteamine may displace cysteine bound in disulfide linkages to proteins, resulting in greater conversion of cysteine to methionine. In addition, methionine is an excellent substrate for the neutral amino acid carrier across the blood–brain barrier [49]. Possibly, the cysteamine treatment results in stimulation of this neutral amino acid transporter or alternatively, egress of methionine from the brain is inhibited.

3.3.3. Thialysine in cerebrum and cerebellum

As noted in the introduction, brain contains appreciable amounts of cystathionine β-synthase and low levels of cysteamine dioxygenase. Given these considerations, conversion of cysteamine to hypotaurine and taurine in the brain will be limited and, therefore, an alternative pathway for metabolism of cysteamine may operate in the brain (Fig. 1). As already mentioned, cysteamine is an alternative substrate to homocysteine for cystathionine β-synthase [1]. The catalytic product generated in the β-substitution reaction catalyzed by human cystathionine β-synthase is thialysine [serine (or cysteine) + cysteamine → thialysine + H_2O (or H_2S)] [1]. As is evident from this equation, if serine is a substrate, the eliminated product is H_2O. If cysteine is a substrate, H_2S is the eliminated product. H_2S is now regarded as a neuromodulator and cerebrovascular messenger [50,51]. Although H_2S may contribute to brain damage induced by stroke [52], this compound is generally regarded as neuroprotective at low doses [50,53] by releasing bound sulfur in neurons and astrocytes in the presence of reductants such as GSH and cysteine [54]. The protective effect of cystamine in Huntington disease mice administered cystamine [47] may also be due in part to the

Table 3
Concentration (pmol/mg of protein) of cysteine, methionine and thialysine in cerebrum and cerebellum at various times following gavage feeding of cysteamine to rats.

Hrs	Cysteine		Methionine		Thialysine	
	Cerebrum	Cerebellum	Cerebrum	Cerebellum	Cerebrum	Cerebellum
"0"	898 ± 166	477 ± 50	193 ± 51	132 ± 9	1.5 ± 1.4	NMA
2	659 ± 83	523 ± 51	212 ± 135	250 ± 61	0.9 ± 0.9	25 ± 25
6	532 ± 96	513 ± 106	829 ± 348	460 ± 105	33.4 ± 0.5	NMA
12	607 ± 10	584 ± 36	927 ± 140	498 ± 23	22.1 ± 0.5	159 ± 6
24	604 ± 14	797 ± 81	240 ± 91	312 ± 82	NMA	85 ± 8

NMA, no measurable amount (<0.2 nmol/mg of protein). $N = 3$.

stimulation of cystathionine β-synthase-catalyzed production of H_2S.

If cysteamine is indeed an alternative substrate for cystathionine β-synthase in the brain, then L-thialysine [*S*-(2-aminoethyl)-L-cysteine, AEC] should be present in the brains of the cysteamine-treated rats. As mentioned in the introduction, we have been unable to find any reports of baseline levels of thialysine in mammalian brain. However, we could detect small amounts of thialysine in the rats administered cysteamine (Table 3).

3.3.4. AECK-DD in rat brain

A previous report suggests that 2H-1,4-thiazine-5,6-dihydro-3-carboxylic acid, the cyclic ketimine derived from transamination of thialysine (trivial name aminoethylcysteine ketimine, AECK), is present in bovine brain at a concentration of 2–3 nmol/g wet weight [15]. A compound derived from AECK by dimerization and decarboxylation and given the trivial name AECK decarboxylated dimer (AECK-DD) has been reported to be present in bovine cerebellum [19]. AECK-DD has been found in human urine [17], human plasma [34,55], human cultured monocytic cells [55] and in dietary vegetables, particularly garlic, asparagus and onion [56].

In the present work we showed that AECK-DD is present in the brains of cysteamine-treated rats (Table 4; Fig. 3). The levels are about 20–40 pmol/mg protein. Assuming that the brain contains 100 mg of protein per g wet weight, then these values translate to about 2–4 nmol/g wet weight of AECK-DD in rat brain. These values are of the same order of magnitude as those reported for bovine cerebellum of 0.6–1.0 nmol/g wet weight [19]. AECK-DD is very hydrophobic and is very efficiently extracted in chloroform from brain homogenates. Interestingly, AECK-DD is the only redox-active species that we can detect in the chloroform extract under the conditions of our HPLC procedure (Fig. 3B).

The original discoverers of the presence of AECK-DD in brain suggested that a pathway for its formation has not been established and that it may be of dietary origin [57]. However, we believe that, based on this group's previous work [19], an endogenous biochemical pathway is also feasible (Fig. 1). Because AECK-DD is derived from thialysine, we had expected that, after administration of cysteamine to rats, the level of this compound would rise in the brain in tandem with increases in thialysine or perhaps be unaltered (if the turnover rate of the compound is slow). Surprisingly, however, the level of AECK-DD dropped by hour twelve (Table 4). A possible explanation is that transamination of thialysine was inhibited in the brains of the cysteamine-treated animals. Another possibility is that the cysteamine treatment caused an oxidative stress in the brain that resulted in a depletion of AECK-DD. In this regard, the compound has been reported to be a more potent anti-oxidant than GSH or ascorbate and on par with α-tocopherol, presumably due to its high lipid solubility (reviewed in [21,57]). Moreover, the compound has been reported to quench reactive oxygen and nitrogen species *in vitro* [34,58–60] and to protect human low-density lipoprotein and a human monocytic cell line against oxidative stress [21]. We note that the potential required to maximally remove an electron from (i.e., oxidize) AECK-DD starts at ~175 mV and is maximal at 225 mV (Fig. 3). Thus, AECK redox capacity is lower than that required to oxidize GSH (which starts at 300 mV and is maximal at 450 mV) but is somewhat higher than that of ascorbate

Table 4
Concentration (pmol/mg of protein) of AECK-DD in cerebrum at various times following gavage feeding of cysteamine to rats.

Hrs	Cerebrum
AECK-DD	
"0"	19, 33
2	34.6 ± 3.2
6	22.6 ± 5.6
12	39.9 ± 7.8
24	4.8 ± 1.6

$N = 3$ at each time point except for the "0" time point where $N = 2$. The cerebellum was not analyzed.

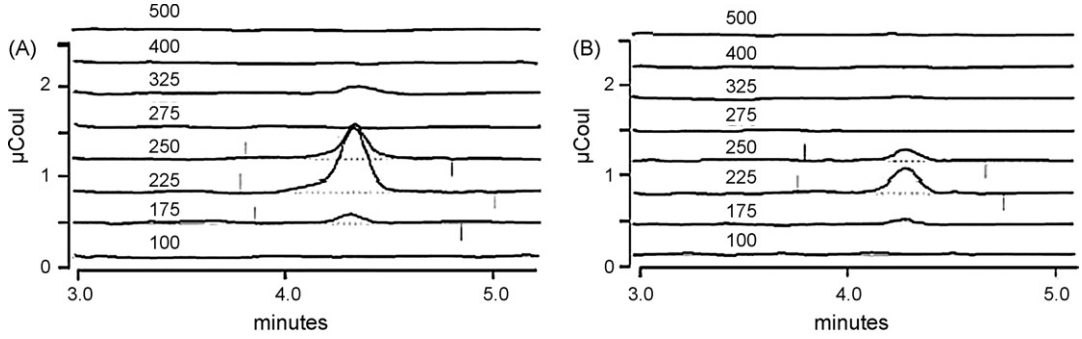

Fig. 3. Detection of AECK-DD in rat brain cerebrum. (A) AECK-DD standard (2 nmol/mL) has a retention time of 4.28 min. AECK-DD is oxidized from 175 to 250 mV with greatest amount of oxidation occurring at 225 mV. (B) AECK-DD extracted from the cerebrum of a rat gavage-fed cysteamine and sacrificed at 0 time (control). The 8-channel CoulArray detectors were set at 100, 175, 225, 250, 275, 325, 400 and 500 mV, respectively. Recovery of authentic AECK-DD in a spiked brain homogenate was consistently about 97%. Note that identification of AECK-DD in the rat brain cerebrum is based on both its identical retention time and its identical redox profile relative to authentic AECK-DD. Note also that AECK-DD is the only redox active compound detectable in the chloroform-extracted cerebrum.

(which starts at ~75 mV and is maximal at 100 mV). Oxidative modifications of AECK-DD that have been described include oxidation to a sulfoxide [61], oxidative dimerization [62] and dehydrogenation [57]. Thus, AECK-DD may be an especially useful antioxidant in the brain because of its highly hydrophobic nature and several functional moieties that are susceptible to oxidation.

We were unable to detect AECK-DD in standard rat chow, but were able to reproduce the finding of Macone et al. [56] that garlic contains appreciable amounts of this compound (data not shown). In a separate experiment, we analyzed the brains of untreated adult male Sprague–Dawley rats (i.e., rats that had not been gavage-fed cysteamine) for thialysine and AECK-DD. We were unable to detect either thialysine or AECK-DD in these brain samples (limit of detection <0.2 nmol/mg of protein). Thus, it appears that AECK-DD may originate in the diet, but that the brain has the capacity to synthesize thialysine and AECK-DD when supplied with an adequate amount of cysteamine. The practicality of these findings is highlighted by dietitians who advocate consumption of vegetables to provide a steady supply of potential antioxidants.

4. Conclusions

The present work demonstrates the usefulness of HPLC coupled to CoulArray (coulometric) detection for the determination of redox-sensitive sulfur-containing compounds in tissues and body fluids. In particular, we have provided analytical methods for those interested in studying cysteamine/cystamine and their potential metabolic products. These analytical procedures may be of interest to researchers focused on the biochemical events associated with the ulcerogenic properties of cysteamine and the neuroprotective effects of pharmacological doses of cystamine. An especially important aspect of our work is the verification of previous workers' findings [18] that, under certain conditions, the brain can accumulate a highly lipophilic substance (AECK-DD) that may be an important antioxidant within a lipid milieu. Further studies on the origin of AECK-DD in brain and its role as an antioxidant in that organ are warranted.

Acknowledgments

The work reported herein was supported in part by the National Institutes of Health grants 2PO1 AG14930 and CA111842, and by funds from the Department of Veterans Affairs, Medical Research Service Merit Review.

References

[1] G. Pitari, G. Maurizi, V. Flati, C.L. Ursini, L. Spera, S. Duprè, D. Cavallini, Biochim. Biophys. Acta 1116 (1992) 27.
[2] G. Pitari, F. Malergue, F. Martin, J.M. Philippe, M.T. Massucci, C. Chabret, B. Maras, S. Duprè, P. Naquet, F. Galland, FEBS Lett. 483 (2000) 149.
[3] R.M. Coloso, L.L. Hirschberger, J.E. Dominy, J.I. Lee, M.H. Stipanuk, Adv. Exp. Med. Biol. 583 (2006) 25.
[4] R.P. Holmes, H.O. Goodman, Z.K. Shihabi, J.P. Jarow, J. Androl. 13 (1992) 289.
[5] T. Togawa, A. Ohsawa, K. Kawanabe, S. Tanabe, J. Chromatogr. B: Biomed. Sci. Appl. 704 (1997) 83.
[6] P. Kubalczyk, E. Bald, Electrophoresis 29 (2008) 3636.
[7] J.T. Pinto, J.M. Van Raamsdonk, B.R. Leavitt, M.R. Hayden, T.M. Jeitner, H.T. Thaler, B.F. Krasnikov, A.J.L. Cooper, J. Neurochem. 94 (2005) 1087.
[8] R.A. Garcia, L.L. Hirschberger, M.H. Stipanuk, Anal. Biochem. 170 (1988) 432.
[9] J. Ogony, S. Mare, W. Wu, N. Ercal, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 843 (2006) 57.
[10] K. Kuśmierek, R. Głowacki, E. Bald, Anal. Bioanal. Chem. 382 (2005) 231.
[11] M.W. Duffel, D.L. Logan, D.M. Ziegler, Methods Enzymol. 143 (1987) 149.
[12] D.K. Rassin, G.E. Gaull, J. Neurochem. 24 (1975) 969.
[13] S. Yu, K. Sugahara, J. Zhang, T. Ageta, H. Kodama, M. Fontana, S. Duprè, J. Chromatogr. B 698 (1997) 301.
[14] M. Nardini, G. Ricci, L. Vesci, L. Pecci, D. Cavallini, Biochim. Biophys. Acta 957 (1988) 286.
[15] M. Nardini, R.M. Matarese, L. Pecci, A. Antonucci, G. Ricci, D. Cavallini, Biochem. Biophys. Res. Commun. 166 (1990) 1251.
[16] D. Cavallini, G. Ricci, S. Duprè, L. Pecci, M. Costa, R.M. Matarese, B. Pensa, A. Antonucci, S.P. Solinas, M. Fontana, Eur. J. Biochem. 202 (1991) 217.
[17] R.M. Matarese, A. Macone, A. Maggio, D. Cavallini, J. Chromatogr. B 683 (1996) 269.
[18] R.M. Matarese, A. Macone, G. Crescentini, S. Duprè, D. Cavallini, Neurochem. Int. 32 (1998) 365.
[19] R.M. Matarese, A. Macone, M. Fontana, S. Duprè, D. Cavallini, Biochem. Mol. Biol. Int. 46 (1998) 829.
[20] M. Nardini, A. Macone, R.M. Matarese, J. Chromatogr. B 795 (2003) 319.
[21] A. Macone, R.M. Matarese, V. Gentili, A. Antonucci, S. Duprè, M. Nardini, Free Radic. Res. 39 (2004) 705.
[22] C. Berruyer, F.M. Martin, R. Castellano, A. Macone, F. Malergue, S. Garrido-Urbani, V. Millet, J. Imbert, S. Duprè, G. Pitari, P. Naquet, F. Galland, Mol. Cell Biol. 24 (2004) 7214.
[23] L. Di Leandro, B. Maras, M.E. Schininà, S. Duprè, I. Koutris, F.M. Martin, P. Naquet, F. Galland, G. Pitari, Free Radic. Biol. Med. 44 (2008) 1088.
[24] C. Berruyer, L. Pouyet, V. Millet, F.M. Martin, A. LeGoffic, A. Canonici, S. Garcia, C. Bagnis, P. Naquet, F. Galland, J. Exp. Med. 203 (2006) 2817.
[25] G. Sarnelli, R. De Giorgio, F. Gentile, G. Calì, I. Grandone, A. Rocco, V. Cosenza, R. Cuomo, G. D'Argenio, Dig. Liver Dis. 41 (2009) 185.
[26] C. Roisin-Bouffay, R. Castellano, R. Valéro, L. Chasson, F. Galland, P. Naquet, Diabetologia 51 (2008) 1192.
[27] T.M. Jeitner, J.T. Pinto, B.F. Krasnikov, M. Horswill, A.J.L. Cooper, J. Neurochem. 109 (Suppl. 1) (2009) 160.
[28] H. Selye, S. Szabo, Nature 244 (1973) 458.
[29] S. Szabo, Am. J. Pathol. 93 (1978) 273.
[30] T. Khomenko, X. Deng, Z. Sandor, A.S. Tarnawski, S. Szabo, Biochem. Biophys. Res. Commun. 317 (2004) 121.
[31] T. Khomenko, S. Szabo, X. Deng, M.R. Jadus, H. Ishikawa, K. Osapay, Z. Sandor, L. Chen, Am. J. Physiol. Gastrointest. Liver Physiol. 290 (2006) G1211.
[32] R. Kleta, W.A. Gahl, Expert Opin. Pharmacother. 5 (2004) 2255.
[33] S. Kim, Z. Zhang, C. Sarkar, P. Tsai, Y. Lee, L. Dye, J. Clin. Invest. 118 (2008) 3075.
[34] A. Antonucci, L. Pecci, R. Coccia, M. Fontana, D. Cavallini, Amino Acids 7 (1994) 83.
[35] S. Szabo, S. Reichlin, Endocrinology 109 (1981) 2255.
[36] L.M. Lichtenberger, S. Szabo, J.S. Trier, Gastroenterology 73 (1977) 1305.
[37] S. Szabo, E.S. Reynolds, L.M. Lictenberger, L.R. Haith Jr., V.J. Dzau, Res. Commun. Chem. Pathol. Pharmacol. 16 (1977) 311.
[38] R.S. Adler, G.T. Gallagher, S. Szabo, Dig. Dis. Sci. 288 (1983) 716.
[39] S. Szabo, H.C. Horner, H. Maull, J. Schnoor, C.C. Chiueh, M. Plakovits, J. Pharmacol. Exp. Ther. 240 (1987) 871.
[40] L.M. Lichtenberger, S. Szabo, E.S. Reynolds, Gastroentoerlogy 73 (1977) 1072.
[41] T. Peters Jr., Adv. Protein Chem. 37 (1985) 161.
[42] J.E. Biaglow, R.W. Issels, L.E. Gerweck, M.E. Varnes, B. Jacobson, J.B. Mitchell, A. Russo, Radiat. Res. 100 (1984) 298.
[43] T.M. Jeitner, D.A. Lawrence, Toxicol. Sci 63 (2001) 57.
[44] A.J.L. Cooper, W.A. Pulsinelli, T.E. Duffy, J. Neurochem. 35 (1980) 1242.
[45] R. Dringen, B. Hamprecht, Neurosci. Lett. 259 (1999) 79.
[46] V. Vitvitsky, M. Thomas, A. Ghorpade, H.E. Gendelman, R. Banerjee, J. Biol. Chem. 281 (2006) 35785.
[47] J.H. Fox, D.S. Barber, B. Singh, B. Zucker, M.K. Swindell, F. Norflus, R. Buzescu, R. Chopra, R.J. Ferrante, A. Kazantsev, S.M. Hersch, J. Neurochem. 91 (2004) 413.
[48] G.W. Douglas, R.A. Mortensen, J. Biol. Chem. 222 (1956) 581.
[49] W.H. Oldendorf, J. Szabo, Am. J. Physiol. 230 (1976) 94.
[50] H. Kimura, Y. Nagai, K. Umemura, Y. Kimura, Antioxid. Redox. Signal 7 (2005) 795.
[51] C.W. Leffler, H. Parfenova, J.H. Jagger, R. Wang, J. Appl. Physiol. 100 (2006) 1065.
[52] K. Qu, C.P.L.H. Chen, B. Halliwell, P.K. Moore, P.T.-H. Wong, Stroke 37 (2006) 889.
[53] M. Lu, L.F. Hu, G. Hu, J.S. Bian, Free Radic. Biol. Med. 45 (2008) 1705.
[54] M. Ishigami, K. Hiraki, K. Umemura, Y. Ogasawara, K. Ishii, H. Kimura, Antioxid. Redox Signal 11 (2009) 205.
[55] R.M. Matarese, A. Macone, R. Antonini, A. Maggio, A. Antonucci, J. Chromatogr. B 732 (1999) 137.
[56] A. Macone, M. Nardini, A. Antonucci, A. Maggio, R.M. Matarese, J. Agric. Food Chem. 50 (2002) 2169.
[57] A. Macone, A. Caiazzo, A. Antonucci, I. Fochi, M. Nardini, S. Duprè, R.M. Matarese, J. Nat. Prod. 70 (2007) 1046.
[58] L. Pecci, G. Montefoschi, A. Antonucci, D. Cavallini, Physiol. Chem. Physiol. NMR 27 (1995) 223.
[59] M. Fontana, L. Pecci, A. Macone, D. Cavallin, Free Radic. Res. 29 (1998) 435.
[60] R.M. Matarese, A. Macone, G. Crescentini, S. Duprè, D. Cavallini, Biochem. Mol. Biol. Int. 46 (1998) 829.
[61] L. Pecci, A. Antonucci, F. Pinnen, D. Cavallini, Amino Acids 18 (2000) 61.
[62] L. Mannina, S. Viel, S. Duprè, L. Pecci, M. Fontana, F. Pinnen, A. Antonucci, A. Segre, Tetrahedron 60 (2004) 4151.