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Spectrophotometric Analysis of the Cyanide Metabolite 2-Aminothiazoline-4-Carboxylic Acid (ATCA)

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Methods of directly evaluating cyanide levels are limited by the volatility of cyanide and by the difficulty of establishing steady-state cyanide levels with time. We investigated the measurement of a stable, toxic metabolite, 2-aminothiazoline-4-carboxylic acid (ATCA), in an attempt to circumvent the challenge of directly determining cyanide concentrations in aqueous media. This study was focused on the spectrophotometric ATCA determination in the presence of cyanide, thiocyanate (SCN^-), cysteine, rhodanese, thiosulfate, and other sulfur donors. The method involves a thiazolidine ring opening in the presence of p-(hydroxy-mercuri)-benzoate, followed by the reaction with diphenylthiocarbazone (dithizone). The product is spectrophotometrically analyzed at 625 nm in carbon tetrachloride. The calibration curve was linear with a regression line of $Y = 0.0022x$ ($R^2 = 0.9971$). Interference of cyanide antidotes with the method was determined. Cyanide, thiosulfate, butanethiosulfonate (BTS), and rhodanese did not appreciably interfere with the analysis, but SCN^- and cysteine significantly shifted the standard curve. This sensitive spectrophotometric method has shown promise as a substitute for the measurement of the less stable cyanide.

Keywords Cyanide Metabolite, 2-Aminothiazoline-4-Carboxylic Acid, ATCA, 2-Iminothiazolidine-4-Carboxylic Acid, ITCA, Spectrophotometric Analysis

INTRODUCTION

Determination of cyanide or its metabolites in biological fluids is necessary for forensic, clinical, military, research, and veterinary purposes. Hydrogen cyanide's (HCN) volatility and nucleophilic nature (Troup and Ballantyne 1987; McMillan and Svoboda 1982) make it difficult to accurately determine concentrations of HCN in biological matrices directly. HCN is rapidly depleted from blood, generally within the first 20 min of exposure (Baskin and Brewer 1997; Moriya and Hashimoto 2001; Sylvester et al. 1981). In addition to rapidly decreasing concentrations of HCN in biological fluids, cyanide sometimes forms as an artifact of storage conditions in a variety of biological samples, including blood (Seto 1996; Ballantyne 1977, 1987; Curry et al. 1967; Sunshine and Finkle 1964).

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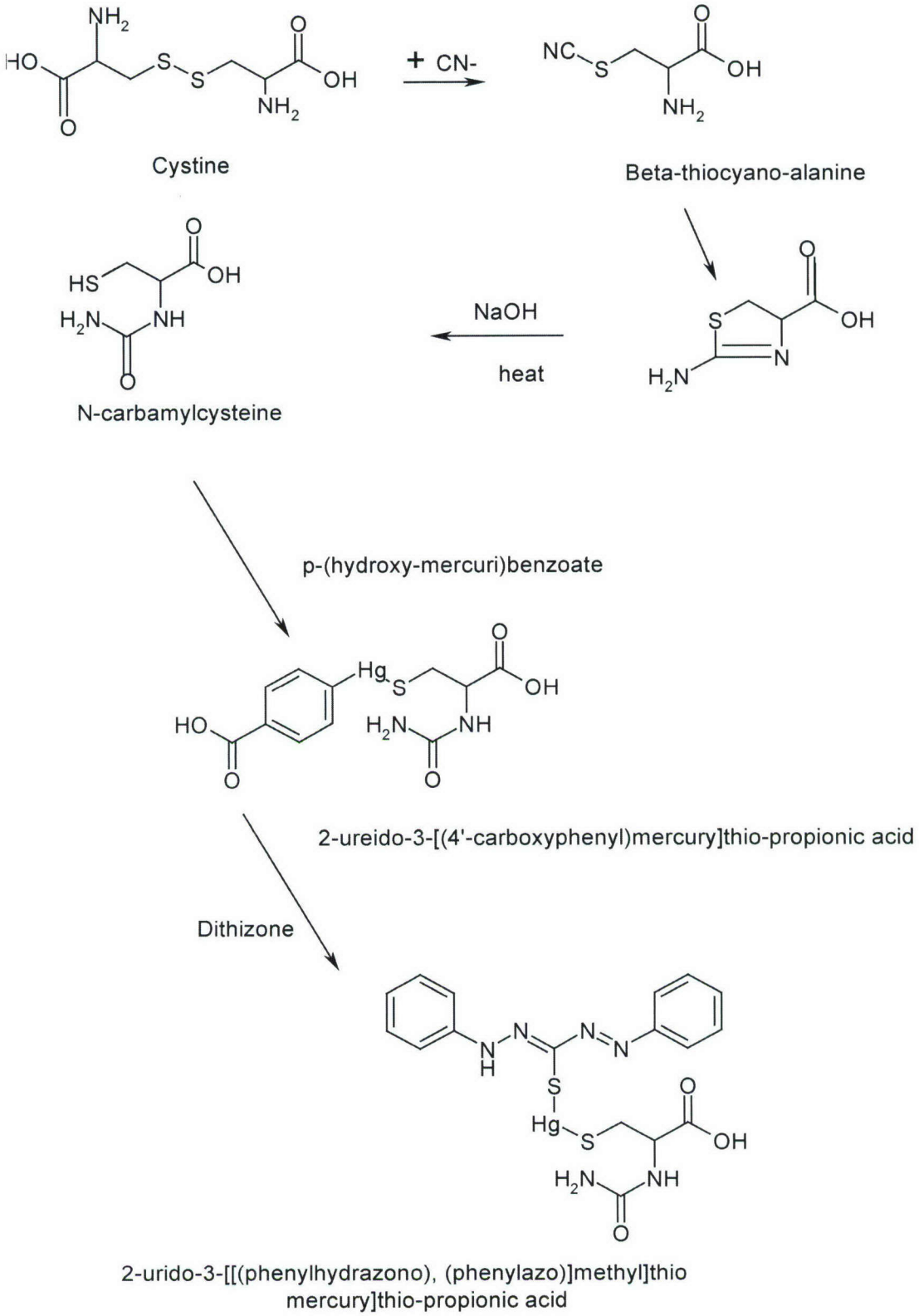

FIG. 1. ATCA formation and subsequent degradation and derivatization reactions used to analyze ATCA.

Common metabolites of HCN include thiocyanate (SCN^-) and 2-aminothiazoline-4-carboxylic acid (ATCA). Most of the investigative effort on the metabolism of cyanide has focused on the formation of SCN^-, the major metabolite of cyanide, in the presence of sulfur donors. This process is catalyzed by the mitochondrial enzyme, thiosulfate (cyanide sulfurtransferase (EC 2.8.1.1)), which is also known by its trivial name of rhodanese (Himwich and Saunders 1948). Under some conditions, other detoxification mechanisms may also become important for cyanide metabolism. For example, chronic administration of cyanide depletes sulfur donors preventing the formation of SCN^-, which may increase the importance ATCA as an alternative biotransformation pathway (Baskin et al. 2004; Isom and Baskin 1997; Lundquist et al. 1995). Another important condition that may shift cyanide metabolism in favor of ATCA is the acidosis that accompanies HCN intoxication. The optimal pH for rhodanese is 8.5, so as bodily pH becomes more acidic, the formation of ATCA may become the major pathway of cyanide metabolism.

ATCA is formed when cyanide reacts with cystine and is present as a tautomer between itself and 2-imino-thioazolidine-4-carboxylic acid (ITCA) (Lundquist et al. 1995). Metabolism of cyanide to ATCA accounts for approximately 20% of cyanide metabolism and increases as cyanide toxicity increases (Baskin and Brewer 1997). ATCA is stable for months in biological samples at freezing and ambient temperatures (Lundquist et al. 1995). Detection of ATCA, a stable cyanide metabolite, can serve as a suitable method to monitor cyanide toxicity and circumvent the disadvantages of other analytical methods. Earlier studies demonstrated the effectiveness of the ITCA determination method to establish cyanide exposure levels (Lundquist et al. 1995). Developing a simple GC-MS method (Logue et al. 2004, 2005) provided a tool for determining elevated ITCA levels in human urine, comparing smokers to nonsmokers for both male and female.

In this study, a simple spectrophotometric method to determine aqueous concentrations of ATCA is investigated with and without common cyanide antidotes present. The method involves a thiazolidine ring opening in the presence of p-(hydroxy-mercuri)-benzoate, followed by the reaction of diphenylthiocarbazone (dithizone) (Fig. 1).

MATERIALS AND METHODS

ATCA Synthesis

ATCA was prepared from L-cysteine (Nagasawa et al. 2004). $NaHCO_3$ (2.8 g, 33 mmol) and cyanamide (1.4 g, 33 mmol) were added to L-cysteine hydrochloride (anhydrous, 5.2 g, 33 mmol) dissolved in 50 mL deionized H_2O. The mixture was heated under reflux and a continuous nitrogen gas flush was maintained for 8 h. Water was occasionally added to replace the evaporative loss, and the pH of entrained gases was monitored at the condenser outlet with Instachek 1-14 pH papers. The pH remained consistent at 10 to 11 (apparently reflecting evolution of gaseous NH_3) until the reaction was nearly complete, at which time it decreased to ~8 to 9, and reflux was discontinued. The reaction mixture was concentrated on a rotary evaporator to approximately 25% of the original volume when crystals spontaneously precipitated. Following overnight refrigeration, the precipitate was collected (0.98 g) and the filtrate was evaporated to a dry, white residue. The residue was dissolved in a few milliliters of hot H_2O and upon cooling produced 1.38 g of crystals. The solids were combined, recrystallized from H_2O/ethanol, 1:5, and dried over P_4O_{10} to yield 1.89 g (39%) of white crystals, mp 225 to 235°C (decomposition). Melting points were taken on a hot-stage melting point apparatus and are uncorrected. Thin layer chromatography was performed using silica gel GF Uniplates from Analtech (Analtech, Inc., Newark, DE) (TLC (n-butanol/acetic acid/H_2O, 4:1:1) $R_f = 0.30$) and reaction products visualized by fluorescence quenching under short-wave UV light and by exposure to iodine vapor in an iodine chamber.

Analysis ($C_4H_6N_2O_2S$): (cal/found) C = 32.87/32.95; H = 4.14/4.07; N = 19.17/19.02. $[\alpha]_D23$ −96.9 (c 1.04, H_2O). Microanalysis was performed by M-H-W Laboratories, Phoenix, AZ.

Synthesis of Butanethiosulfonate (BTS)

BTS was prepared as a sodium salt from butanesulfonyl chloride and sodium sulfide by the method of Traeger and Linde (1901) with minor modifications. Sodium sulfide ($Na_2S{\cdot}9H_2O$) (45.2 mmol; 10.88 g) was dissolved in deionized distilled water (40 mL) maintained at 95 to 100°C; butanesulfonyl chloride (45.2 mmol) was slowly added, and the reaction mixture was refluxed with stirring for 10 to 12 h. The solvent was removed with a rotating evaporation in vacuo, and hot ethanol was added to dissolve the product from the solid residue and thereby separate it from sodium chloride. The crude product was purified by repeated recrystallization from 95% ethanol and subsequent chromatography on a silica gel column (70–230 mesh, 60 Å, 5 μm spherical, 2.5 × 32.0 cm) obtained from Aldrich Chemical Co, Inc. (Milwaukee, WI) with a solvent system of ethylacetate:methanol:water (6:3:0.05 v/v). BTS was prepared for chromatography by dissolving it in 95% ethanol and mixing it with 10% silica. After solvent removal, the dry mixture of silica and BTS was carefully layered on the top of the column and was developed with the eluent solvent system. The chemical reaction was monitored by thin layer chromatography, and the purity was determined by HPLC analysis, proton NMR, and reactive sulfur assay. The HPLC spectra were recorded on a Rainin Gradient HPLC system with a Hitachi UV spectrophotometric detector, using a Brownlee column (spherical silica, 5 μm, 0.46 × 10 cm) with amino polar phase that was obtained from Rainin Instrument Co, Inc. (Woburn, MA). Proton NMR spectra were recorded on a Varian Gemini spectrometer for D_2O solution (δ scale, DSS as internal standard) (200 MHz).

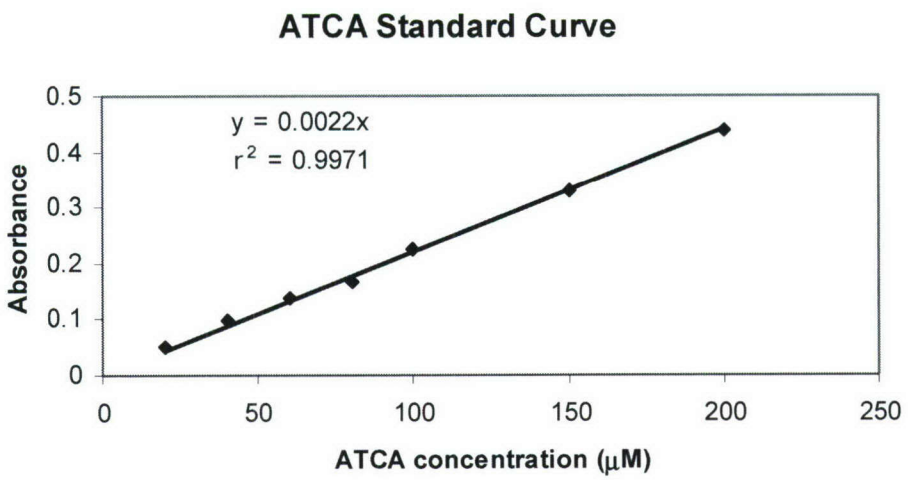

FIG. 2. Calibration curve of ATCA (0–200 μM). Linear correlation of the data (y = 0.0022x) gave a correlation coefficient of 0.9971.

Preparation of Rhodanese, Rhodanese Activity Determination

Bovine liver rhodanese was purified as described previously (Leung et al. 1986) using the method of Westley (1981). In addition, the enzyme was further purified by size exclusion chromatography (Sephacryl S-200 Pharmacia LKB Biotechnology, Piscataway, NJ). Formation of thiocyanate was measured spectrophotometrically at periodic intervals as described by Westley (1981) using the method of Mintell and Westley (1966). Rhodanese activity was determined by suspending an aliquot (10–100 μl) in a phosphate buffer solution containing 50 mM KCN and 50 mM $Na_2S_2O_3$. One unit of rhodanese (RU) is designed as the amount of enzyme that catalyzes the production of 1 μmol of thiocyanate/min at 22°C.

ATCA Analysis

The method of Brandham et al. (1965) was slightly modified for this study. Briefly, to a sample containing 500 μl of 20 to 200 nmol/mL ATCA, 500 μl each of 400 μM p-(hydroxymercuri)benzoate reagent (in 0.1 M phosphate buffer, pH = 7.0) and 0.5 mL of 2.0 M NaOH were added. The sample solutions were heated to boiling for 1 h. After cooling, 200 μl of 3.2 M citric acid solution was added to buffer the solution to pH = 6. Five milliliters of diphenylthiocarbazone(dithizone) reagent (40 μg/mL in CCl_4, diluted threefold freshly before use) was added and the mixture was vortexed vigorously for 60 sec. After the two phases separated, the absorbance of the organic phase was measured at 625 nm on a Shimadzu UV-2101 PC spectrophotometer.

To determine the viability of the spectrophotometric method for the determination of ATCA concentrations in the presence of various compounds associated with cyanide poisoning or cyanide antidotes, the ATCA concentration in the assay solution was kept constant (100 μM) and the concentrations of the substances were varied from 10 to 250 μM. The absorbance was monitored as the concentration of these possible interfering compounds was increased.

RESULTS

Figure 2 shows the standard curve for the ATCA. The absorbance changed linearly with the amount of ATCA within the concentration range studied (0–200 μM). Each data point represents the arithmetic mean of three independent measurements. The equation for the linear regression line is y = 0.0022x, with a correlation coefficient of 0.9971. The relative percentage deviation (RPD) for each data point was less than 10%. It is shown (Fig. 2) that the sensitivity to ATCA is considerable in this assay.

To determine the effects of various cyanide antidotes and other potential interferents on this analysis method, the ATCA concentration was kept constant (100 μM) and the concentrations of the test compounds thiosulfate, BTS, rhodanese, cysteine, cyanide, and SCN^- were varied. In Figures 3 and 4, the ratio of ATCA in the presence of increasing concentrations of each test compound is given relative to ATCA alone. For example, the absorbance of 100 μM ATCA in the presence of increasing concentrations of thiosulfate is given as relative absorbance. Relative absorbance = absorbance of (ATCA + thiosulfate)/absorbance of ATCA alone. Each point was determined as a result of three measurements and expressed as the arithmetic mean ±% CV, and is graphed as a percentage, with 100% indicating no interference was detected.

Thiosulfate did not interfere with the ATCA determination. The linear fit gave a slightly positive slope of 0.055. The slope of the fitting line with the BTS was also slightly positive (0.059). The relative absorbance decreased with increasing rhodanese concentration, giving a definite negative slope for the final fitted curve (slope = –0.144). The relative absorbance curve showed a maximum at the cysteine concentration of 30 μM, but at higher cysteine concentrations there were no notable changes in the absorbance. Cyanide did not show any remarkable effects on

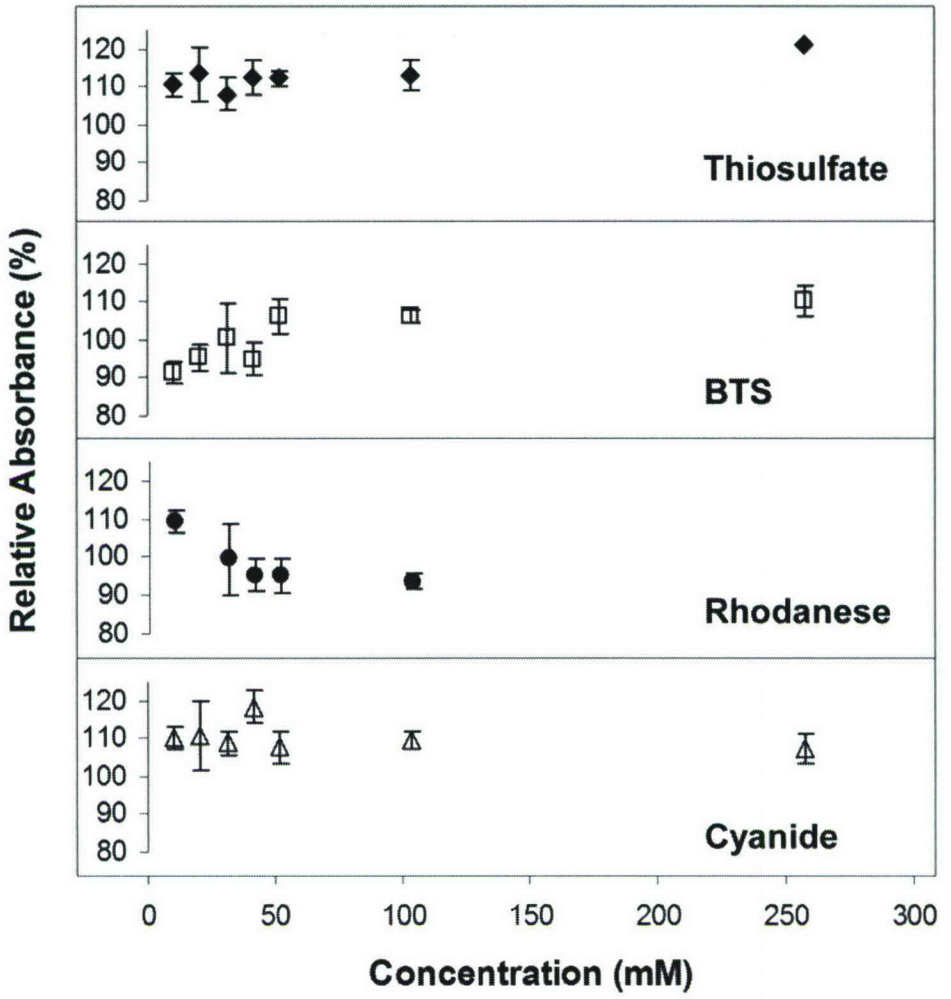

FIG. 3. Relative absorbance of ATCA with increasing concentrations of thiosulfate, BTS, cyanide, and rhodanese. These substances were found to not interfere with ATCA determination by the spectrophotometric method.

the absorbance. SCN^- produced the most remarkable changes in the relative absorbance curve. A linear regression analysis of the data for the cysteine and SCN^- gave r^2 values of 0.33 and 0.37, respectively, which suggests that the linear fit is inadequate for the data shown in Figure 4.

DISCUSSION

The major metabolic product of cyanide is SCN^-, especially in the liver, where plenty of rhodanese is present to catalyze the conversion of cyanide to the less toxic SCN^- (Isom and Baskin 1997). Therefore, cyanide does not have remarkable liver toxicity. The major target organs for cyanide toxicity are the brain and heart. In these organs the rhodanese level is low; therefore, the SCN^- formation is suppressed, and the formation of ATCA becomes important. It is known that ATCA is a neurotoxic agent causing hippocampal damage (Bitner et al. 1997). ATCA is formed from cystine and it is present in equilibrium with the 2-iminothiazolidine-4-carboxylic acid (ITCA) form. At pH = 7.4 it is mainly present as ATCA (Nagasawa et al. 2004). To determine the ATCA distribution in the body it was necessary to determine how the other substances, which are participating in one of the metabolic pathways in the body, interfere with ATCA measurements. Lundquist (1995) published an HPLC method to analyze ATCA, but that method involves a derivatization step, making it cost, time, and labor intensive. The present method is relatively sensitive, simple, and fast. The standard curve gave a good correlation ($r^2 > 0.99$) using p-(hydroxy-mercuri)-benzoate and diphenylthiocarbazone (dithizone) reagents.

Thiosulfate, BTS, rhodanese, and cyanide did not significantly interfere with the analysis, as all absorbance measurements were within a 10% RPD. In this study, both cysteine and SCN^- interfere, requiring further studies to determine the mechanism. Based on these studies, the present ATCA analysis method seems to be a suitable method for the measurement of the cellular and tissue distribution of ATCA. This should contribute to an understanding of the mechanism of the toxicity

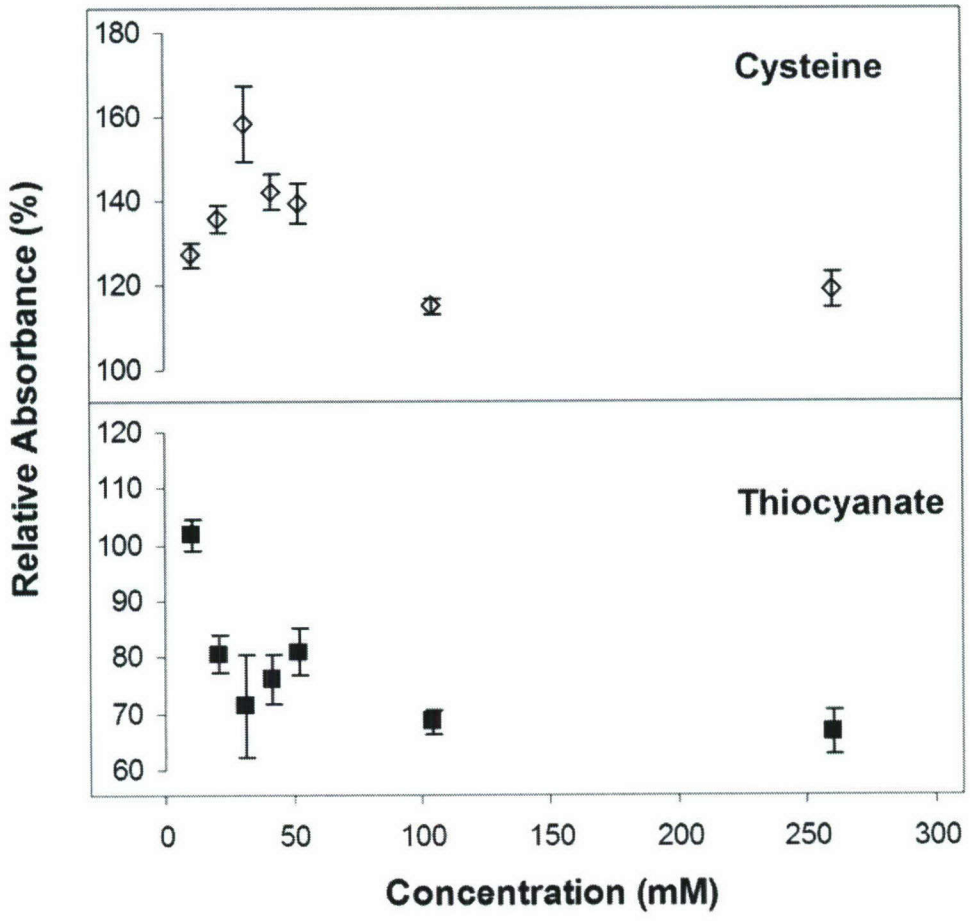

FIG. 4. Relative absorbance of ATCA with increasing concentrations of thiocyanate and cysteine. Both thiocyanate and cysteine interfere with the analysis of ATCA by this method.

of ATCA, and of cyanide itself, in an aspect different from the study of cyanide alone. Although the toxicity of ATCA has rarely been studied, it is known that it has excitotoxic properties, which can cause hippocampal damage (Bitner et al. 1997). Death occurred following convulsions after CNS ATCA injection (A. Kanthasamy, personal communication). Therefore, besides the possibility of using ATCA as an improved marker of cyanide intoxication, if ATCA is found to play a major role in cyanide-induced neurotoxicity, antagonism of ATCA may serve as a tool against cyanide toxicity. This spectrophotometric method could serve as a substitute for the measurement of the less stable cyanide in tissues where SCN^- and cysteine are not accumulated in appreciable concentrations or following sample preparation by solid-phase extraction. Development of a more sensitive (Fig. 2) and specific analytical technique is necessary to determine the potential of ATCA as a replacement for cyanide determination in biological matrices. In addition, while the interaction of cystine and cyanide to form ITCA and ATCA accounts for approximately 20% of cyanide metabolism, and increases with cyanide dosage (Baskin and Brewer 1997), correlation between in vivo ATCA levels and cyanide dosing will require tissue studies. However, this method has the potential to allow for a simpler approach to the measurement of a metabolite that is more stabile than cyanide.

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