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Simultaneous determination of cysteine sulfinic acid and hypotaurine in rat tissues by column-switching high-performance liquid chromatography with electrochemical detection

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Abstract

Cysteine sulfinic acid (CSA) and hypotaurine (HT) were determined by electrochemical detection with a glassy carbon electrode at 0.95 V vs. Ag/AgCl. The separation of CSA and HT was accomplished by coupled-column liquid chromatography, consisting of an anion-exchange column and a cation-exchange column. For the determination of CSA and HT in rat tissues, a column-switching system was introduced to remove interferences from late-eluting endogenous substances. The limits of determination were 0.05 μM for both sulfinic acids. The average precisions (C.V.) over the concentration range of 0.05 to 5 μM were 4.3% for CSA and 4.2% for HT. © 1997 Elsevier Science B.V.

Keywords: Sulfinic acids; Cysteine sulfinic acid; Hypotaurine

1. Introduction

Several metabolic pathways generating sulfinic acids (RSO_2H) are known, but the biological roles of the sulfinic acids in vivo are not clearly understood. Only cysteine sulfinic acid (CSA) and hypotaurine (HT) have been detected in tissues of normal animals [1], and homocysteinesulfinate has been measured in the urine of patients with homocystinuria [2]. Two additional sulfinic acids, β-sulfinopyruvate and β-sulfinoacetaldehyde, are formed in vivo but spontaneously decompose with the loss of SO_2 [3]. CSA and HT are catabolites in the biosynthesis of taurine from cysteine. CSA has a similar structure to aspartate, and Curtis and Watkins [4] showed that it had a strong excitatory action in the spinal cord. Moreover, CSA stimulates the formation of cyclic AMP in the guinea pig cerebral cortex in vitro [5]. CSA is converted to HT by the action of CSA decarboxylase (EC 4.1.1.29), and HT is the immediate precursor of taurine biosynthesis and acts as a superoxide scavenger and a sperm mobility factor [6,7].

To study the physiological significances of the sulfinic acids, a sensitive analytical method which can detect nanomolar concentrations is needed. Several analytical methods for the determination of CSA, HT and the related compounds in biological samples have been reported such as gas chromatography (GC) [8,9], high-performance liquid chromatography (HPLC) [10–12] and an enzymatic cycling method [13]. However, some of the published meth-

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ods involve time-consuming steps such as solid-phase extraction (SPE) for sample clean-up, or a derivatization procedure for detection. To avoid the time-consuming procedure for sample clean-up, a column-switching technique was incorporated into the present method, which allows direct injection of the supernatant after the deproteinization into the chromatographic system. Furthermore, our work shows that the electrochemical activity of sulfinic acids allows a sensitive determination by an amperometric method using a glassy carbon electrode as the working electrode. The objective of the work described here was to develop a sensitive method for the simultaneous determination of CSA and HT in biological samples.

2. Experimental

2.1. Chemicals

L-Cysteine sulfinic acid monohydrate and hypotaurine were obtained from Sigma (St. Louis, MO, USA). Citric acid monohydrate and lithium hydroxide were of amino acid analytical grade and were purchased from Wako (Osaka, Japan). All the other chemicals were of analytical grade, and obtained from the usual commercial sources.

2.2. Instrumentation

Cyclic voltammograms were measured with a cyclic voltammetric analyzer VM010 (Yanagimoto, Tokyo, Japan) equipped with a glassy carbon electrode and an Ag/AgCl (3.3 *M* KCl) electrode as the reference electrode. The chromatographic system consisted of three L-6000 pumps (Hitachi, Tokyo, Japan), a Rheodyne Model 7125 injector with a 50 μl sample loop, two SPV-OS-6 six-port valves (GL Science, Tokyo, Japan), an LC amperometric detector E-502 (IRICA, Kyoto, Japan) with a glassy carbon electrode. A Chromatocorder 11 (Sic Instruments, Tokyo, Japan) was used for data processing.

2.3. Chromatographic conditions

The overall scheme of the chromatographic system is presented in Fig. 1. For pre-column 1 (PC1, 50 mm×4 mm I.D.) and the separator column 2 (SC2, 150 mm×4 mm I.D.), Nucleosil 5SB (anion-exchange resin, Macherey-Nagel, Duren, Germany) was used as the packing material, and pre-column 2 (PC2, 30 mm×4 mm I.D.) and separator column 1 (SCl, 50 mm×4 mm I.D.) were packed with TSK gel SCX (cation-exchange resin, Tosoh, Tokyo, Japan). The mobile phase for pumps 1, 2 and 3 was 70 m*M* lithium citrate buffer (pH 2.50). Prior to use, the mobile phase was degassed and filtered through a 0.2 μm Millipore filter. Each pump delivered the mobile phase at a flow-rate of 0.3 ml/min. The switching times for valves V1 and V2 are listed in Table 1. Electrochemical detection was carried out at 0.95 V vs. Ag/AgCl (3.3 *M* KCl).

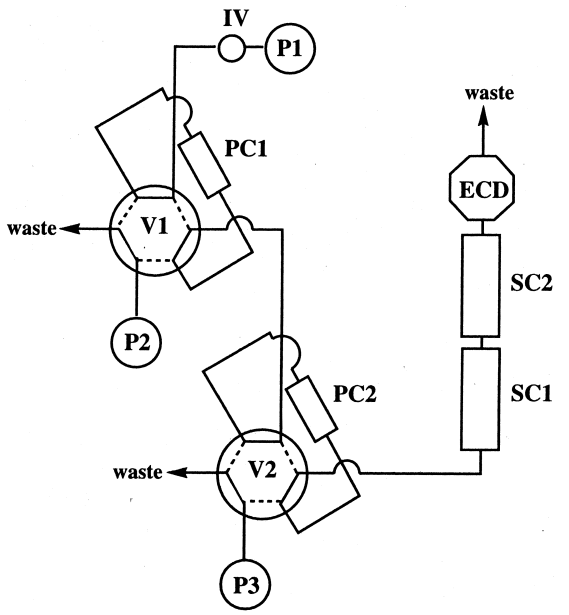

Fig. 1. Scheme of the column-switching chromatographic system. P1, P2 and P3=pump; IV=injection valve; V1 and V2=switching valve; PC1 and PC2=pre-column; SC1 and SC2=analytical column; ECD=electrochemical detector. PC2 and SC1, TSK gel SCX; PC1 and SC2, Nucleosil 5SB.

2.4. Sample preparation

Male Wistar rats (weight: 260–300 g) were killed by decapitation and the tissues were immediately removed on ice. Tissue was homogenized in 2.5

Table 1
Time program for the column-switching system

Time after injection (min)	Switch Valve No.	Event
0.0		The sample is injected onto PC1
8.0	V1	PC1 is disconnected and back-flushed to remove the anionic late-eluting components
13.5	V2	PC2 is disconnected and back-flushed to remove the cationic late-eluting components
40.0	V1 and V2 reset	After the chromatogram is completed, the next sample is injected

volumes of ice-cold 70 m*M* lithium citrate buffer (pH 2.50), and 2.5 volumes of 10% 5-sulfosalicylic acid were added to the homogenate. The homogenate was centrifuged at 7000 *g* for 10 min at 4°C. The supernatant was filtered through a 0.2 μm filter and an aliquot of the filtrate was applied to the HPLC system.

3. Results and discussion

3.1. Electrochemical detection of sulfinic acids

Sulfinic acids in aqueous solution are easily oxidized to the corresponding sulfonic acids by the addition of hydrogen peroxide. However, to our knowledge, no paper has been published on the HPLC determination of the sulfinic acids using electrochemical detection. Cyclic voltammograms were measured in order to check the electrochemical behavior of the sulfinic acids for their detection. Both CSA and HT were irreversibly oxidized at 0.86 V vs. Ag/AgCl in 0.05 *M* Britton–Robinson buffer (pH 2~pH 8). Fig. 2 shows the cyclic voltammograms of CSA and HT at pH 5. According to the chromatographic conditions established below, hydrodynamic voltammograms of CSA and HT were obtained. The response of the electrochemical detection for sulfinic acids increased with increasing applied potentials, but the baseline of the chromatogram became unstable at the potential of above 1.0 V. CSA and HT can be simultaneously detected at 0.95 V with a relatively good signal-to-noise ratio.

3.2. Separation of CSA and HT

The separation of CSA and HT in a standard mixture using the cation-exchange resin was examined. HT was separated by changing the pH of the citrate buffer, but CSA was eluted at the void volume. CSA is too acidic to be retarded by the sulfonic groups of the cation-exchange resin. CSA was retained when the strongly anion-exchange resin was used, however, HT passed through the column in the void volume. Therefore, two columns, an

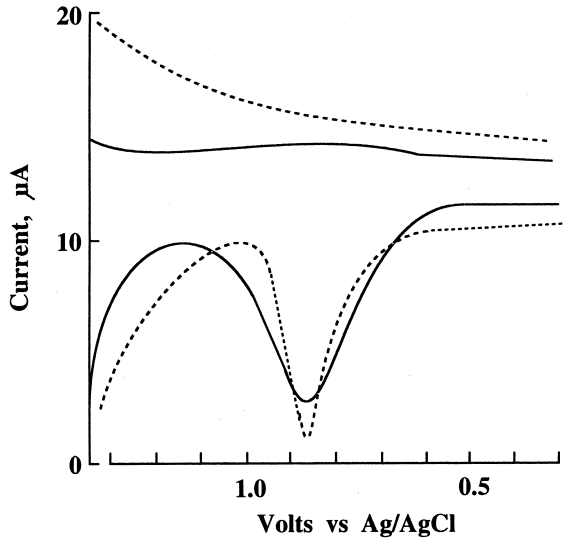

Fig. 2. Cyclic voltamograms of cysteine sulfinic acid and hypotaurine in Britton–Robinson buff (pH 5). Initial potential, 0.3 V; scan rate, 100 mV/s; sample concentration, 1 m*M*. Solid line, CSA; broken lin, HT.

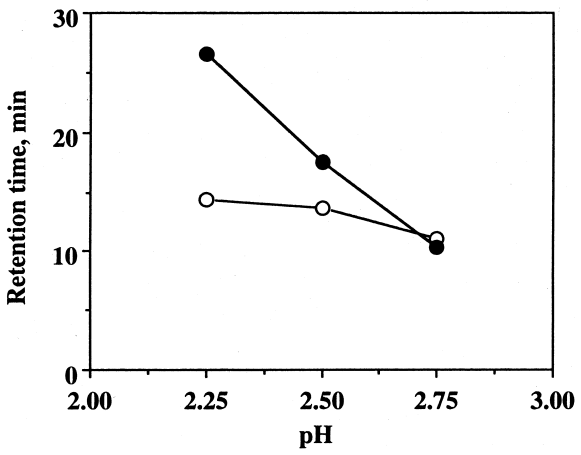

Fig. 3. Effect of pH on the retention time of cysteine sulfinic acid (○) and hypotaurine (●) using the two ion-exchange column systems. Eluent: 50 m*M* lithium citrate buffer; flow-rate, 0.5 ml/min; column, TSK gel SCX (50×4 mm I.D.)+Nucleosil 5SB (150×4 mm I.D.).

anion and cation-exchange column combined in series, were used as the separator columns. Fig. 3 shows the effect of the pH on the retention of CSA and HT under the two-column system. Both sulfinic acids were well separated at pH 2.50.

3.3. Column-switching system for biological samples

In the preliminary studies, the deproteinizing supernatant was directly injected onto a coupled-column system. Under such conditions, many peaks due to the endogenous compounds appeared on the chromatogram for a long time. For the elimination of late-eluting compounds, pretreatments using several mini-cartridges are required. However, multiple clean-up procedures are not adequate for CSA and HT because the sulfinic acids are unstable against autooxidation. Therefore, a column-switching system was introduced to remove interferences from the late-eluting endogenous components. In order to establish the conditions of column-switching, the time required for elution of CSA and HT was investigated using only pre-columns. The elution times from the pre-columns (PC1, PC2) were 8 min for CSA and 13 min for HT. To construct the system using one switching valve, simultaneous elution from the pre-columns is required for CSA and HT. However, the volume of PC2 (30×4 mm I.D) can not be reduced due to reproducibility of column packing. Using the other cation-exchange resin for PC2, HT could not be successfully fractionated from interfering substances present in the tissue extracts. Therefore, as shown in Fig. 1, PC1, PC2 and two separation columns (SC1+SC2) were combined with two switching valves. After the sulfinic acids were transferred to the separator column, the pre-columns were back-flushed to remove the components of the matrix that were strongly retained. The switching-valve positions used during the chromatography and the functions of these positions are presented in Table 1. The recovery of the column-switching event was close to 100% for both sulfinic acids and was calculated by comparing the peak area of a standard solution after injection into the coupled-column system with the same amount directly injected onto the column. After repeated injections of the biological samples into the column-switching system, no interfering peak was observed in the chromatograms, suggesting that late-eluting compounds were removed.

3.4. Validation

Calibration curves for CSA and HT in aqueous solution obtained using the column-switching method were linear between 0.05 and 20 μ*M* with correlation coefficients of 0.999. The limits of detection were 0.5 pmol for both sulfinic acids in a 25 μl injection volume. The within-day variability was determined by performing replicate analyses ($n=5$) of an aqueous standard mixture. The coefficient of variation (C.V.) from the concentrations of 0.05, 0.5, 5.0 μ*M* were 4.0, 4.4 and 4.6%, respectively, for CSA and 8.9, 1.9 and 1.8%, respectively, for HT.

3.5. Determination of CSA and HT in rat tissues

Recovery studies were performed using tissue homogenates spiked with known amounts of CSA and HT. 5 μ*M* each of CSA and HT was added to the 20% (w/v) homogenates of brain, liver and kidney. The recoveries for CSA and HT ranged from 91 to 105% with a C.V. of 4.4–7.4% ($n=5$). In addition, the recovery for CSA was carried out at a spiked concentration of 0.91 μ*M* in the same man-

ner. The recoveries for brain, liver and kidney were 92.0±4.02, 89.8±5.24 and 87.0±2.46%, respectively (n=5).

Using the present method, brain, liver and kidney samples were analyzed for CSA and HT. A representative chromatogram of rat kidney is shown in Fig. 4. No interfering peaks were observed in the chromatograms. The peaks eluted before CSA showed weakly ionic character but we could not identified. The concentrations of CSA and HT in rat tissues are given in Table 2. Only a few reports of CSA and HT tissue levels are available in the literature. The values of CSA for rat tissues in the literature [10,11] are not the same. Ida and Kuriyama [10] reported the CSA content in rat brain by HPLC based on *o*-phthalaldehyde detection. Their value was approximately ten-times higher than the value obtained in this study, although the reason for such a difference is not known at present. On the other hand, it has been reported that CSA was not detected in rat brain, liver and kidney [11], and our values for these tissues were around the limit of the detection for CSA using their methods. We also determined CSA and HT in rat tissues by the current method based on pre-column derivatization with *o*-phthalaldehyde [11]. CSA in all tissues and HT in the brain were not detected. The mean (±S.D.) concentrations of HT in the liver and kidney were 39.7±15.4 and 192.2±22.7, respectively (n=5). These values are in the same range as the results obtained by the present method.

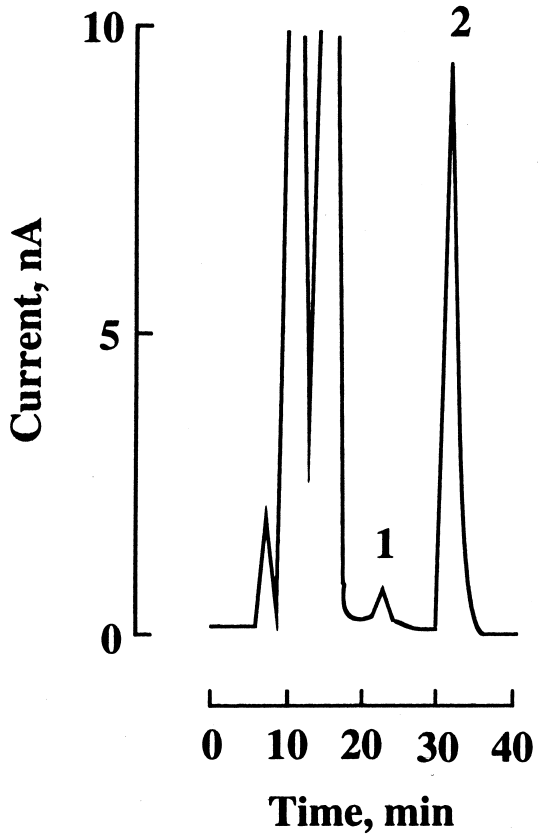

Fig. 4. Chromatogram of cystein sulfinic acid and hypotaurine in rat kidney. Peaks: 1=CSA; 2=HT. The analytical conditions are given in Section 2.3.

Table 2
Determination of cysteine sulfinic acid and hypotaurine in rat tissues

Tissue	Cysteine sulfinic acid (nM/g)	Hypotaurine (nM/g)
Brain	2.7±2.5	34.9±11.2
Liver	0.15±0.30	32.5±15.7
Kidney	6.1±3.8	153.6±82.9

n=5, mean±S.D.

4. Conclusions

A HPLC column-switching method with electrochemical detection for the simultaneous determination of CSA and HT in rat tissues has been developed. The column-switching system could eliminate time-consuming clean-up procedures. The sensitivity of this method was at least ten-times higher than that of the amino acid analyzer with *o*-phthalaldehyde detection. It should be useful for the measurement of CSA and HT in biological samples. Using a computer with a HPLC-manager, it is possible to control the electric switching valves in order to automate the method.

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