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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF 2-AMINOETHYL HYDROGEN SULFATE IN PLASMA AND URINE

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SUMMARY

A sensitive and specific method for the analysis of 2-aminoethyl hydrogen sulfate in biological fluids by high-pressure liquid chromatography is described. Aliquots from deproteinized plasma or from acidified urine were subjected to ion-exchange clean-up by eluting with water through two columns packed with Dowex 1-X4 (Cl^- form) and Dowex 50W-X8 (H^+ form) resins. Recoveries in the eluates were consistent and quantitative (95–100%). Effluents from such treatment were chromatographed on a Durrum cation-exchange column with 0.05 *M* citrate buffer containing 0.15 *N* Na^+ (pH 2.6). Detection was accomplished with an automated fluorescamine detection system. Using 2 ml of plasma or urine, 2-aminoethyl hydrogen sulfate could be assayed at concentrations of 0.3 μg/ml. Linear responses were observed up to at least 133 μg/ml. The method is both accurate and reproducible with a relative standard deviation of about $\pm$ 4.0%.

INTRODUCTION

2-Aminoethyl hydrogen sulfate (AEHS) has been found at Abbott Laboratories to have diuretic activity in animals. In order to examine its bio-availability and pharmacokinetic characteristics, it was necessary to have an analytical method for the determination of its concentrations in biological fluids. After several unsuccessful attempts to make derivatives for gas chromatographic analysis, development of a method based on high-pressure liquid chromatography (HPLC) was undertaken.

Since AEHS has negligible ultraviolet (UV) absorption, precluding the use of a UV photometer as detector, design of a sensitive detection method was studied. A relatively new reagent, 4-phenyl-spiro [furan-2(3H),1′-phthalarn]-3,3′-dione, known as fluorescamine, has been shown to form highly fluorescent products with primary amines[1,2]. The reaction proceeds almost instantaneously at room temperature at alkaline pH. Both the reagent itself and its hydrolysis products are non-fluorescent. These properties make the reagent ideally suited for use in automated detection systems. Design of such a detector as part of an amino acid analyzer has been described[3]. It has been claimed to be two orders of magnitude more sensitive for the

detection of picomole quantities of amino acids than the standard ninhydrin method. Recently, a fluorescamine detector for the analysis of diamines has also been reported[4].

In the present study, an automated fluorescamine detection system was constructed and attached to a high-pressure liquid chromatograph. It was found to be highly sensitive for detection of AEHS. A method for the analysis of AEHS in plasma and urine utilizing such HPLC system has been developed, and described in this report.

EXPERIMENTAL

Reagents and materials

Unless otherwise stated, all chemicals were analytical reagent grade and were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.), AEHS, taurine (2-aminoethane sulfonic acid), and phenol were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Fluorescamine (Fluram™) was from Hoffmann-La Roche (Nutley, N.J., U.S.A.). Dowex 1-X4 anion-exchange resin (50–100 mesh, Cl^- form), Dowex 50W-X8 cation-exchange resin (200–400 mesh, H^+ form) and disposable columns were purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.).

Solutions were prepared as follows. Eluent: citric acid monohydrate (10.5 g, 0.05 *M*), 9.95 g of sodium sulfate (0.07 *M*) and 1 g of phenol were dissolved in distilled water containing 1.0 ml of aqueous 10 *N* sodium hydroxide solution and diluted to 1000 ml (pH 2.6). Borate buffer: the pH of a 0.16 *M* boric acid solution was adjusted to 9.8 by titrating with concentrated sodium hydroxide solution. Fluorescamine solution: fluorescamine (40 mg) was dissolved in 200 ml of acetone. Column regenerating solution: ethylenedinitrilotetraacetic acid disodium salt (0.25 g) was dissolved in 1 l of 0.3 *N* sodium hydroxide.

Chromatographic system

Fig. 1 is a schematic flow diagram of the chromatographic system used. Three Model M-6000 pumps from Waters Assoc. (Milford, Mass., U.S.A.) were used to deliver eluent, borate buffer, and fluorescamine solution. The mobile phase was pumped through a Model 720 sample injection valve equipped with a 25-μl sample loop (Micromeritics, Norcross, Ga., U.S.A.) into a 48-cm stainless-steel column packed with cation-exchange resin (Durrum, Palo Alto, Calif., U.S.A.; Cat. No. 18837). The effluent was then passed through coil A (*ca.* 40 cm) into the mixing device where the fluorescent derivatives were formed. Stainless-steel tubing (1/16 in. O.D., 0.009 in. I.D.) was used for all the coils. Coils B (70 cm) and C (150 cm) served as additional pulse dampeners for the pumps. Air displacing the fluorescamine solution in the reservoir was passed through 50% sulfuric acid in a trap as described by Stein *et al.*[3]. A chromatographic column (2.4 × 30 cm) with a 10-μm porosity fritted glass filter (Glenco Scientific, Houston, Texas, U.S.A.) was used as a reservoir for fluorescamine solution. All inlet tubings from reservoirs to pumps were 1/8 in. O.D. (0.125 in. I.D.) Teflon®. Two 3-way ball valves (Whitey, Oakland, Calif., U.S.A.; Cat. No. 41 x S2), as shown in Fig. 1, were installed for convenience in replacing fluorescamine solution with acetone at the end of a day's operation. This was done to prevent fluorescamine deposition within the pump. A Swagelok® union cross (Crawford Fitting, Solon, Ohio, U.S.A.), which was drilled to form 1/16 in. I.D. orifices allowing the four tubings to join end to end, was used as a low dead-volume mixing device. Coil D

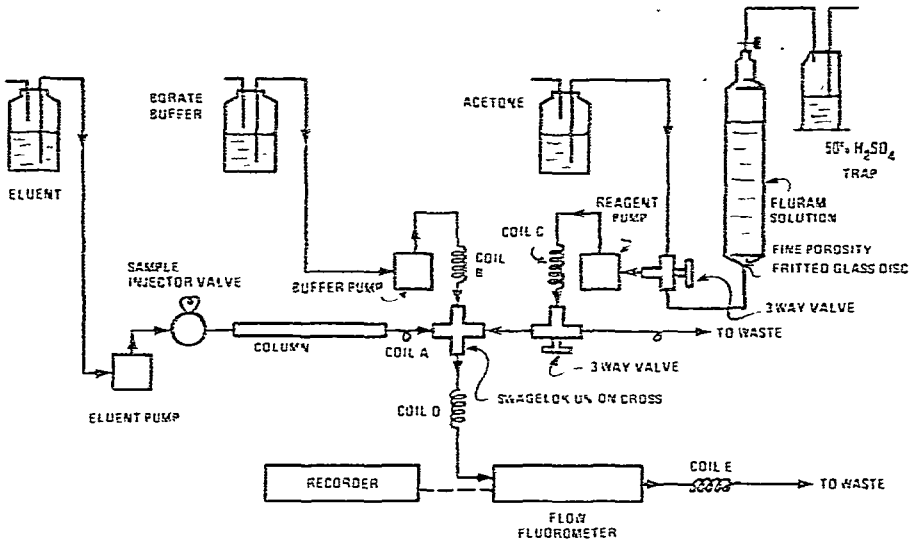

Fig. 1. Schematic illustration of the high-pressure liquid chromatographic system.

(250 cm) allowed approximately 16 sec of reaction time at a total flow-rate of 0.4 ml/min (*ca.* 10 sec at 0.6 ml/min). A shorter tubing (100 cm) was also found satisfactory at a total flow-rate of 0.4 ml/min. Coil E (150 cm) was installed to provide sufficient back pressure to prevent the formation of bubbles when acetone was mixed with aqueous buffer solutions.

The fluorescent products of the derivatization were detected with a flow fluorometer (Fluoro-Monitor, Model 1309, Laboratory Data Control, Riviera Beach, Fla., U.S.A.). A band of radiant energy with a wavelength range of 320–400 nm was used for excitation. The fluorescence emission (range 400–700 nm) was detected after passing through a UV blocking secondary filter. Each of the flow cells had a volume of *ca.* 10 μl. The response from the Fluoro-Monitor was recorded on a 10-mV Model A-25 Series 9145 strip-chart recorder (Varian Aerograph, Walnut Creek, Calif., U.S.A.).

Chromatographic conditions

Unless otherwise stated, the column was operated at ambient temperature and the flow-rates for eluent, borate buffer and fluorescamine reagent were 0.1 ml/min (1900 p.s.i.), 0.2 ml/min (120 p.s.i.) and 0.1 ml/min (150 p.s.i.), respectively.

Regeneration of the HPLC column was accomplished by ten successive injections of 25 μl of 0.3 *N* aqueous sodium hydroxide at intervals of 20 sec. It was performed as required or after approximately 60 injections of plasma or urine samples.

Procedures

Assay of plasma samples. To an aliquot (2.0 ml) of plasma in a conical centrifuge tube, was added 1.0 ml of 20% trichloroacetic acid (TCA). After being stirred briefly on a Vortex mixer, the mixture was centrifuged for 10 min at *ca.* 1500 *g*, and the supernatant was transferred to a 15-ml screw-cap tube. The aqueous solution was

extracted twice with 3 ml of ether, and 1.5 ml of the aqueous phase transferred on to a Dowex 1-X4 resin column (Cl^- form, 0.7 × 6.0 cm) which was connected to the top of a Dowex 50W-X8 resin column (H^+ form, 0.7 × 6.0 cm). The columns were eluted with 7 ml deionized water, and the eluate collected (*ca.* 8.5 ml) was diluted to 10 ml in a volumetric flask. A 25-μl aliquot of the solution was introduced into the HPLC system and analyzed.

A set of samples prepared from control plasma spiked with AEHS at levels of 5, 50 and 100 μg/ml was prepared and analyzed along with the unknown samples. A calibration curve was constructed from peak heights of the plasma standards *versus* their concentrations. The concentrations of the unknown samples were derived from the curve.

Assay of Urine Samples. An identical procedure was used for urine samples, except that 50 μl of 20% TCA was added to 2.0 ml of urine, and 1.5 ml of the acidified urine was applied directly on to the ion-exchange columns without centrifugation.

RESULTS AND DISCUSSION

Since its introduction in 1972, fluorescamine has found many applications in various assays as a strong fluorogenic reagent for primary amines[2–7]. In all cases, ease of operation and high sensitivity have been claimed. An automated fluorescamine detection system was shown to be relatively easily constructed and to have much higher sensitivity for the analysis of amino acids than the conventional ninhydrin method[2,3]. A similar setup has also been described for the detection of polyamines[4]. In both reported designs, column effluent was mixed with the buffering agent prior to the addition of the fluorescamine solution. In the present study, a Swagelok union cross was used for mixing of column effluent with borate buffer and fluorescamine reagent. This was found to be adequate for the completion of the derivatization reaction. Since low dead volume was found to be essential for high resolution, small bore tubing (0.009 in. I.D.) was used throughout the detection system. A low dead-volume mixing device was made by drilling off the internal shoulders of the union cross to make 1/16 in. I.D. orifices, allowing the four tubes to extend to the center of the union. The system, as illustrated in Fig. 1, is particularly suited for high-resolution liquid chromatography.

AEHS reacts with fluorescamine almost instantaneously in alkaline medium to form a highly fluorescent derivative. Preliminary studies indicated that, at pH 9, the excitation and emission maxima of the fluorophor were 390 nm and 490 nm, respectively. Under the chromatographic conditions specified in Experimental, an AEHS solution gave a single fluorescence peak with a retention volume of about 0.95 ml. No increases of fluorescence responses were observed by increasing the flow-rate or the concentration of fluorescamine solution, or by varying the ratio of buffer to reagent. To test the sensitivity and linearity of such a detection system, a series of aqueous AEHS solutions were analyzed. The lower detection limit was estimated to be about 50 ng/ml, representing approximately 10 picomoles of injected compound. Linear responses were obtained from 0 to at least 10 μg/ml. These observations were comparable to the reported values for amino acids[3]. The fluorescamine detector for the amino acid analyzer was shown to have a lower detection limit of about 20 picomoles. Linear fluorescent responses were observed up to 2.5 nmoles of injected amino

acid. The reproducibility of the fluorometric determination of AEHS by the proposed HPLC method was determined by analysis of eight separately prepared aqueous solutions. The data which reflect both the precision and accuracy of the method are shown in Table I. The relative standard deviation was found to be ± 1.2% indicating good reproducibility and accuracy.

TABLE I

PRECISION OF THE FLUOROMETRIC DETERMINATION OF 2-AMINOETHYL HYDROGEN SULFATE BY HPLC

Concentration of AEHS (μg/ml)	*Relative fluorometric response (arbitrary units)*	*Ratio of response to concentration*
10.0	1668	166.3
10.9	1833	168.2
10.6	1812	170.9
6.46	1104	171.2
10.8	1828	169.3
6.00	992	165.3
6.35	1068	168.2
10.3	1740	168.9
	Mean	169
	Relative standard deviation	± 1.2%

For the assay of AEHS in biological fluids, taurine (which is present in both control plasma and urine) tends to interfere because of its structural similarity to AEHS. Several attempts to separate the two compounds on HPLC using HPLC packings such as C_{18} reversed-phase, strong cation-exchange, and strong anion-exchange resins were unsuccessful. Another approach involving the direct HPLC analysis of the fluorescamine derivatives of AEHS and taurine was also briefly investigated. It was found that the fluorescamine derivatives of both compounds could be chromatographed using a C_{18}/Corasil reversed-phase column. However, the study was discontinued after several attempts to separate the two fluorescent derivatives were unsuccessful.

Aliquots from deproteinized plasma or acidified urine which was not subjected to ion-exchange clean-up could be analyzed for AEHS by the HPLC procedure. However, regeneration of the cation-exchange column was required after injections of about five samples due to presence of other amino acids. Such a practice is time-consuming. Amino acid analysis of the samples which had been subjected to ion-exchange clean-up as described in *Procedures* showed that AEHS and endogenous taurine were the major ninhydrin positive components with only traces of other amino acids. Thus, it was possible to analyze about 60 samples without regeneration of the HPLC column.

Fig. 2 illustrates typical chromatograms obtained with samples prepared from (a) a mixture of AEHS and taurine, (b) a plasma blank, and (c) a plasma sample spiked with AEHS. Samples prepared from control plasma gave only one peak corresponding to endogenous taurine. To the extent of the detection limit (approximately 0.3 μg/ml), AEHS was found not to be a normal constituent of human or dog plasma.

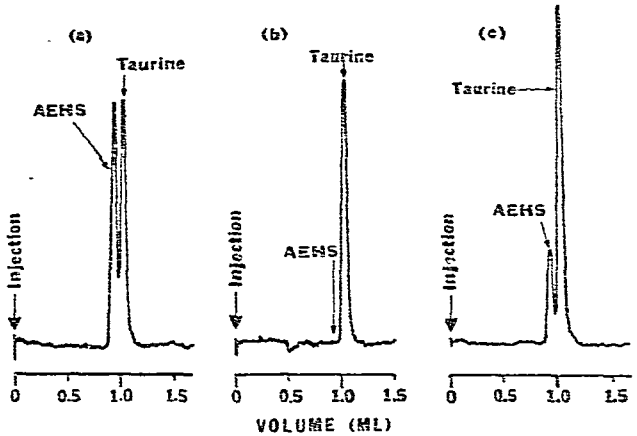

Fig. 2. High-pressure liquid chromatograms of samples prepared from (a) a mixture of AEHS and taurine (*ca.* 13 ng each injected), (b) a plasma blank, (c) a plasma spiked with 2.66 μg AEHS per ml.

Typical retention volumes for AEHS and taurine were 0.95 and 1.05 ml, respectively. The resolution function, *Rs*, was estimated to be 0.85 according to the formula:

$$Rs = 2(t_2 - t_1)/(w_1 + w_2)$$

where $(t_2 - t_1)$ is the difference in retention times for the two adjacent peaks and $(w_1 + w_2)$ represents the sum of baseline peak widths in time units[8].

Recovery of AEHS from the ion-exchange resins was studied. Samples prepared from 4 ml of control plasma to which AEHS had been added at a level of 2.66, 26.6 or 133 μg/ml were deproteinized by mixing with 2 ml of 20% TCA. After centrifugation, the supernatants were transferred to 15 ml screw-cap tubes and the excess TCA was removed by ether extraction (2 × 6 ml). A portion of the aqueous phase (1.5 ml) was treated with ion-exchange resins according to the procedure described in *Assay of plasma samples*. Another 1.5-ml portion of the aqueous solution was pipetted into a volumetric flask and diluted with distilled water to 10 ml. Both samples were subjected to HPLC analysis. The relative peak heights of AEHS from the treated and untreated samples were used to calculate the recovery. Five sets of separately prepared samples were used at each plasma level, and the results are shown in Table II. Recovery was found to be consistent and quantitative (95–100%).

TABLE II

RECOVERY OF 2-AMINOETHYL HYDROGEN SULFATE FROM SPIKED BLANK PLASMA SAMPLES USING ION-EXCHANGE TREATMENT

Sample no.	*% Recovery at concentration indicated*		
	2.66 μg/ml	*26.6 μg/ml*	*133 μg/ml*
1	100.0	99.0	98.0
2	97.3	96.2	96.0
3	96.2	97.2	95.4
4	100.0	98.1	98.1
5	96.0	94.5	95.7
Mean	97.9	97.0	96.6
Relative standard deviation	2.0%	1.8%	1.4%

Fifteen 2-ml plasma samples were prepared, five each at concentrations of 2.66, 26.6, and 133 μg/ml. These were carried through the analytical procedure. The results, as listed in Table III, indicate the method to be reproducible, with a relative standard deviation of ± 4%. A least-squares best fit was calculated for peak heights, y, *versus* the corresponding plasma AEHS concentrations, x. The equation of the resulting line was $y = 17.5\,x + 6.4$. The y intercept of 6.4 was not statistically significantly different from zero. The correlation coefficient was 0.9997, clearly establishing the linearity of the data. When samples from plasma containing 266 μg AEHS per ml were carried through the HPLC analysis, it was necessary to increase the flow-rate of fluorescamine solution to 0.2 ml/min in order to obtain consistent responses. The borate buffer solution was adjusted to 0.3 ml/min so that precipitation of inorganic salts in the tubings could be avoided. Under these conditions, with increased amount of fluorescamine, a slight deviation from linearity was observed at the 266 μg/ml level, suggesting that non-linearity is not caused by insufficient reagent.

TABLE III

ANALYSIS OF 2-AMINOETHYL HYDROGEN SULFATE IN PLASMA

Sample no.	*AEHS concentration (μg/ml)*		*% of theory*
	Theoretical	*Found*	
1	2.66	2.51	94.4
2	2.66	2.61	98.1
3	2.66	2.61	98.1
4	2.66	2.41	90.6
5	2.66	2.41	90.6
Mean	2.66	2.51	94.4
Relative standard deviation			± 4.0%
6	26.6	27.0	101.7
7	26.6	27.3	102.5
8	26.6	27.3	102.5
9	26.6	25.6	96.4
10	26.6	26.8	100.8
Mean	26.6	26.8	100.8
Relative standard deviation			± 2.5%
11	133	131	98.7
12	133	131	98.7
13	133	132	99.0
14	133	138	103.6
15	133	133	100.0
Mean	133	133	100.0
Relative standard deviation			= 2.1%

In the same manner, precision for assay of the drug in urine was studied. Fifteen 2-ml urine samples were prepared, five of each at concentrations of 5.4, 54, and 108 μg/ml. They were analyzed by the HPLC method. The results, as shown in Table IV, indicate good precision of the method. A linear equation relating peak height, y, to the urine concentration, x, was derived from the data, giving $y = 8.15\,x + 11.9$, with a correlation coefficient of 0.9992.

TABLE IV

PRECISION OF HPLC ANALYSIS OF 2-AMINOETHYL HYDROGEN SULFATE SPIKED URINE

Sample no.	*Peak heights (arbitrary units) observed at urine concentrations indicated*		
	5.40 μg/ml	*54 μg/ml*	*108 μg/ml*
1	51	464	904
2	49	460	864
3	50	464	904
4	51	452	872
5	51	472	893
Mean	50.4	462	887
Relative standard deviation	± 1.8%	± 1.6%	± 2.1%

Analysis according to the procedure outlined in Experimental, has a lower detection limit of about 0.3 μg/ml for plasma and urine samples. With increased size of plasma or urine sample, or by concentrating the eluate from ion-exchange clean-up treatment, the detection limit could be improved. The method was used for the analysis of plasma and urine samples collected from animals that had been administered the drug. Presented in Fig. 3 is a typical plasma AEHS profile of a female beagle dog that had been given a 60 mg/kg or 30 mg/kg oral dose of the compound.

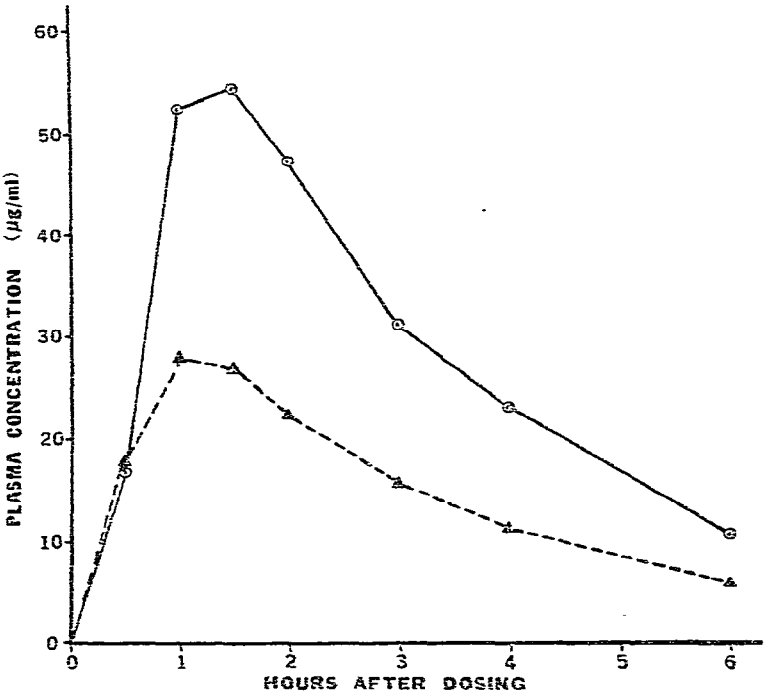

Fig. 3. Dog plasma levels of AEHS following a 60 mg/kg (○—○) or a 30 mg/kg (▲—▲) oral dosing.

In conclusion, a specific and sensitive analytical procedure for AEHS in plasma or urine has been developed utilizing HPLC and the fluorescamine detection system. Since fluorescamine reaction with primary amines is quite general, it should be possible to apply such a detection system for the HPLC analysis of other amino compounds.

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