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SAMPLE PREPARATION PROCEDURE FOR DETERMINATION OF DOPAMINE SULFATE ISOMERS IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DUAL-ELECTRODE ELECTROCHEMICAL DETECTION

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SUMMARY

We developed a procedure utilizing small columns of solid-phase extraction material for sample preparation for the determination of dopamine sulfate (DAS) isomers in human urine. Processed sample is then subjected to high-performance liquid chromatography (HPLC) with dual-series-electrode electrochemical detection. Dopamine 3-O-sulfate (DA-3-S) and dopamine 4-O-sulfate (DA-4-S) were determined using two different HPLC systems The ratio of the urinary excretion rate of DA-3-S to DA-4-S was relatively constant, but the 24-h excretion rates of total DAS varied widely among individuals. This method should prove useful in future studies concerning the metabolic and physiologic roles of DAS isomers.

INTRODUCTION

Dopamine sulfate (DAS), a metabolite of dopamine (DA) [1], may occur as either of two isomers: dopamine 3-O-sulfate (DA-3-S) or dopamine 4-O-sulfate (DA-4-S). DAS accounts for more than 98% of the total DA found in human plasma [2]. It has been suggested that DAS is metabolically active [3] and can be converted to free catecholamines [4, 5]. This conversion may be important in the catecholamine metabolism of exercise-trained animals [6].

Most assays for measurement of endogenous DAS utilize acid or enzymatic hydrolysis to convert DAS to free DA and thus cannot distinguish between the

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two isomers of DAS. This capability may be important since recent work has shown differences in the physiological actions of the two isomers. Racz et al. [7] have shown that DA-3-S, but not DA-4-S, could inhibit the angiotensin-stimulated release of aldosterone in cultured bovine adrenal cells. A number of different workers have measured DAS isomers in the urine of subjects who were given L-DOPA [8—10]. These procedures, which utilize resin-based ion exchangers and ultraviolet detection, are not able to quantify endogenous levels of DAS isomers in human urine. In addition, these procedures require extensive and time-consuming clean-up methods with total processing time ranging from 3.5 h [8] to 57 h [10]. Elchisak and Carlson [11], using similar sample preparation methods, were able to quantify endogenous DA-3-S from urine of normal subjects with ultraviolet detection. DA-4-S was not quantified owing to chromatographic interferences.

Several post-column reaction procedures have been developed which separate DAS isomers by high-performance liquid chromatography (HPLC), hydrolyze DAS isomers to form free DA, and then detect free DA. A procedure developed in our laboratory [12] used an in-stream post-column hydrolysis procedure to convert DAS isomers to free DA; the free DA was then detected by single-electrode electrochemical detection. Arakawa and co-workers [13, 14] were able to measure catecholamine sulfoconjugates in normal human urine utilizing fluorometric detection. This procedure utilized photocleavage of the O-sulfate bond followed by the reaction of free catecholamines with diaminoethane and oxygen. Yamamoto et al. [15] were able to measure DA-3-S and DA-4-S in normal human plasma and urine by taking advantage of the specific reaction of *p*-aminobenzoic acid with DA. DA-3-S and DA-4-S were separated chromatographically, hydrolyzed and determined in a continuous-flow reaction system. Each of these post-column reaction procedures is sensitive and reproducible. They all, however, require relatively complex detection systems which are not available commercially.

A method recently developed in our laboratory utilizing reversed-phase HPLC with dual-series-electrode electrochemical detection can quantify both DA-3-S and DA-4-S isomers in aqueous solutions [16]. Dual-series-electrode electrochemical detection offers greater sensitivity and convenience for the direct detection of DAS isomers when compared to single-electrode detection. However, owing to chromatographic interferences, DAS isomers in urine cannot be measured without extensive sample purification procedures. In addition, lipophilic compounds in the urine are strongly retained in a reversed-phase chromatographic system making analysis times excessively long.

In this paper, we describe a sample preparation method for DA-3-S and DA-4-S from human urine which minimizes the problems of interfering compounds and excessive analysis times by using small reversed-phase and strong anion-exchange (SAX) columns prior to the HPLC analysis of urinary DAS.

EXPERIMENTAL

Sample collection

Urine samples (24 h) were collected from six healthy laboratory employees

(three males and three females) between the ages of 22 and 35. Urine was collected on ice, and sodium bisulfite (25 g/l), EDTA (5 g/l) and 6 *M* hydrochloric acid (40 ml/l) were immediately added. After addition of all preservatives the pH was 2—3. Aliquots were frozen and stored at —70°C until analyzed for DAS isomers.

Sample preparation

Urine samples were thawed and diluted 1:1 with doubly distilled water containing the internal standard, [^{3}H]DAS (approximately 500 cpm per tube). Samples were then processed with a solid-phase extraction (SPE) system (J.T. Baker, Phillipsburg, NJ, U.S.A.). This consisted of a vacuum manifold to which small SPE columns (500 mg of packing material) were attached. We used both reversed-phase (C_{18}) and SAX SPE columns. The C_{18} columns were pretreated by successively washing with approximately 2 ml methanol, water and 0.1 *M* acetate buffer (pH 4.0) containing 1% EDTA. The SAX columns were pretreated by successively washing with approximately 2 ml hexane, methanol, water and 0.1 *M* acetate buffer (pH 4.0) containing 1% EDTA. Diluted urine sample (1 ml) was applied to a C_{18} column. The liquid was then slowly aspirated by vacuum (20 mmHg) until the meniscus was level with the top of the column packing. The column was washed under vacuum (38 mmHg) with two successive 500-μl aliquots of 0.5 *M* acetic acid. DAS was then eluted with two additional 500-μl aliquots of 0.5 *M* acetic acid. The fractions (containing DAS) were combined and then applied to a pretreated SPE SAX column (quaternary amine, acetate form), and aspirated. This column was washed with 750 μl doubly distilled water and DAS was eluted with two 500-μl aliquots of 0.5 *M* acetic acid. The sample was then dried in an evacuated centrifuge (Savant Instruments, Hicksville, NY, U.S.A.), resuspended in 1 ml of appropriate mobile phase, and a portion (50 μl) injected into the HPLC system.

High-performance liquid chromatography

Two different HPLC systems were utilized for the separation of DA-3-S and DA-4-S. Each system was equipped with a stainless-steel column (250 × 4 mm I.D.) prepacked with 5-μm C_{18} silica (Biophase, Bioanalytical Systems, West Lafayette, IN, U.S.A.) and a guard column (20 × 4.5 mm I.D.) packed with 40-μm C_{18} Corasil (Waters Assoc., Milford, MA, U.S.A.). Pressure pulses were dampened by a column of air connected in parallel with the solvent flow path and by increasing the amount of tubing between the pump and the injector on each system.

Two different mobile phases were used. Mobile phase A consisted of 25 m*M* monochloroacetic acid (pH adjusted to 3.0) with 1 m*M* EDTA. *n*-Octylamine (4 m*M*) was added as an ion-pairing agent after pH adjustment [11]. Mobile phase B consisted of 75 m*M* potassium phosphate containing 1 m*M* EDTA (pH 4.8) [17]. For each system, the flow-rate was set at 0.5 ml/min. A dual-electrode electrochemical detector was used [BAS LC-4B/17(D), Bioanalytical Systems]. The dual electrodes were in the series configuration and were modified for fraction collection. The potential was maintained at +0.85 V on the upstream electrode and —0.010 V on the downstream electrode for mobile phase A and +1.00 V and 0.00 V, respectively, for mobile phase B.

All potentials are versus an Ag/AgCl reference electrode. Effluent containing the DAS isomers was collected immediately after the detector and radioactivity measured by liquid scintillation counting (Beckman Instruments, Fullerton, CA, U.S.A.) in appropriate fractions. The measured radioactivity was used to correct for the variation in recoveries observed with the SPE columns.

RESULTS AND DISCUSSION

Optimization of sample preparation procedure

Several eluents were tested to establish the most effective elution procedure for DAS isomers on SPE columns. Fractional recoveries of DAS standards from the C_{18} reversed-phase columns are presented in Table I. Fractional recoveries of standards from the quaternary amine SAX columns are presented in Table II. Neither column, by itself, was able to routinely clean all urine samples. We then decided to use them in series using a combination of eluents. Of the combinations attempted only the procedure described above in the

TABLE I

RECOVERIES OF DAS STANDARDS FROM SOLID-PHASE EXTRACTION C_{18} COLUMNS AS A FUNCTION OF THE ELUTING SOLVENT

Fraction (ml)	Recovery* (%)				
	Acetic acid		1 *M* Tris (pH 7.4)	10% Methanol	0.075 *M* KH_2PO_4 (pH 4.8)
	0.2 *M*	0.5 *M*			
0—1	0	0	0	8	N.D.**
1—2	51.2	89.5***	14.5	87.5	6
2—3	39.2	19.9	35.5	1.5	23.5
3—4	6.5	0	22.5	0	23.5

*Values are the average of both isomers. Approximately 1 nmol of each DAS isomer was used. Recoveries did not differ significantly between isomers. $n = 1$.
**N.D. = not determined.
***$n = 3$.

TABLE II

RECOVERIES OF DAS STANDARDS FROM SOLID-PHASE EXTRACTION SAX COLUMNS WITH VARIOUS ELUTING SOLVENTS

Fraction (ml)	Recovery* (%)					
	0.5 *M* Acetic acid		0.5 *M* Potassium chloride		Water	
	DA-3-S	DA-4-S	DA-3-S	DA-4-S	DA-3-S	DA-4-S
0—1	36.9	16.8	68.2	55.4	43.6	17.2
1—2	39.9	54.7	30.8	41.6	49.4	62.2
2—3	14.3	14.7	0	2.0	3.2	20.6

*Approximately 1 nmol of each DAS isomer was used. $n = 1$.

Experimental section succeeded in giving good, consistent separation of DA-3-S and DA-4-S and relatively short HPLC analysis times for human urines.

Assay validation

The overall recovery of DAS standards carried through the entire procedure was 37.2 ± 2.27% for DA-3-S and 47.0 ± 0.45% for DA-4-S (n = 3). Recovery

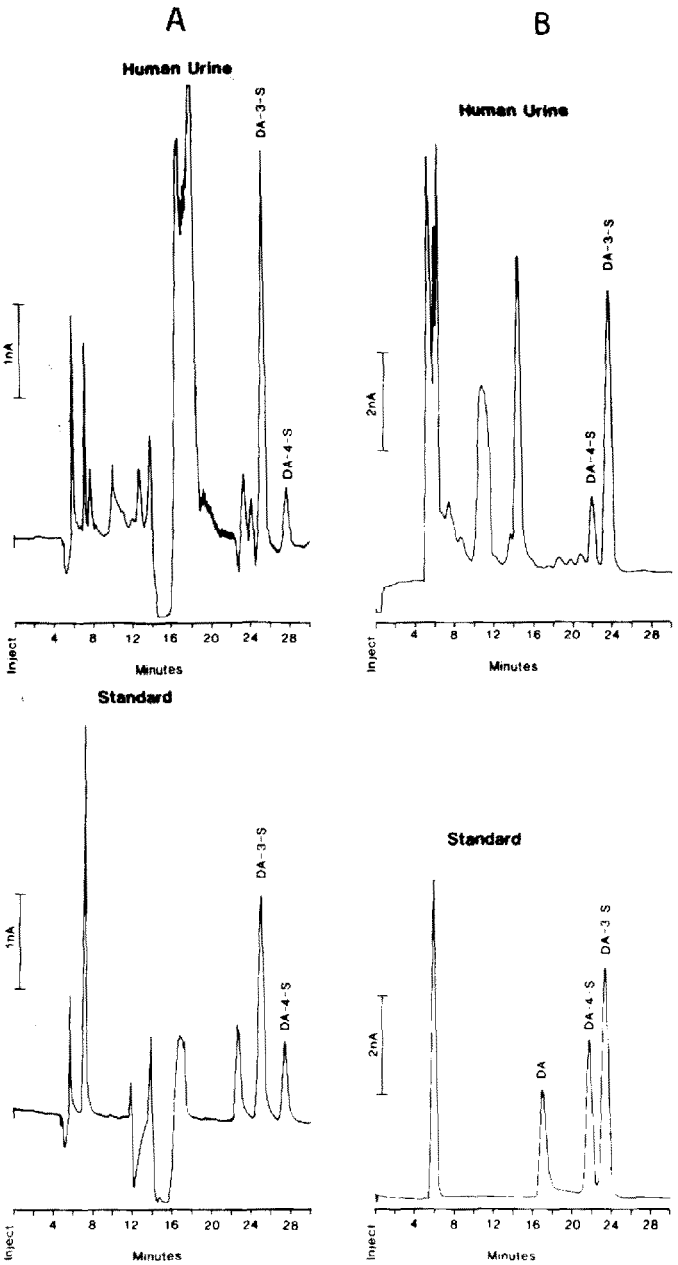

Fig. 1. Chromatograms of DAS standards and processed human urine samples in (A) mobile phase A and (B) mobile phase B.

of both DAS isomers in spiked urine samples was similar to recovery of standards. Using [^{3}H]DAS as an internal standard, standard curves were constructed and were linear over the range tested (approximately (0.1—7 nmol). The within-assay coefficient of variation for DAS concentration in a urine sample carried through the entire determination procedure was 4.0% for DA-3-S and 5.1% for DA-4-S (n = 3). The between-assay coefficient of variation was 10.6% for DA-3-S and 13.6% for DA-4-S (n = 3).

Chromatographic analysis times for urine samples were between 35 and 40 min. One sample could be purified by the SPE columns in approximately 5—10 min. Samples were dried in the evacuated centrifuge in approximately 2 h thus making the overall processing time for ten samples less than 3 h. Eight samples could easily be processed and run on the HPLC system in one working day.

DAS in human urine

Fig. 1A shows chromatograms (mobile phase A) of authentic DA-3-S and DA-4-S standards and a human urine sample after they were processed according to the sample clean-up procedure. Fig. 1B shows the same standards and sample analysed using mobile phase B. The DAS peaks from processed urine sample appear to be free from any significant interferences. The peaks identified as DAS in urine coelute perfectly with DAS standards. Furthermore, urinary DAS concentrations determined for the same samples using the two different HPLC systems correlate well (r values: DA-3-S, 0.999; DA-4-S, 0.988; Fig. 2). These observations support the identification of the DA-3-S and DA-4-S peaks in human urine.

Table III lists daily urinary excretion rates for the six subjects studied. Excluding subject No. 6, these values are similar to those previously reported [11, 14, 15]. While there is a large inter-individual variation in the total DAS measured, the ratio of the DA-3-S excretion rate (or DA-4-S) to the total DAS excretion rate was relatively constant.

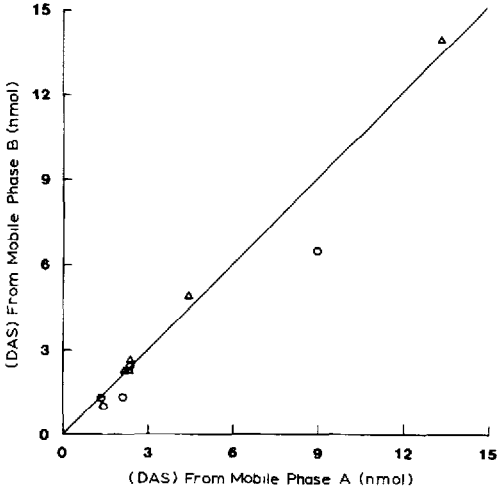

Fig. 2. Correlation between the amounts (nmol/ml of urine) of DAS in the samples determined by the two HPLC separation methods. For DA-3-S (○), r = 0.999; for DA-4-S (△), r = 0.988.

TABLE III

DOPAMINE SULFATE ISOMERS IN HUMAN URINE

Subject	Excretion rate (μmol per day)			Percentage of total DAS	
	DA-3-S	DA-4-S	Total DAS	DA-3-S	DA-4-S
1	1.95	1.04	2.99	65.2	34.8
2	3.02	1.71	4.73	63.8	36.2
3	1.14	0.68	1.82	62.6	37.4
4	1.73	1.67	3.40	50.9	49.1
5	4.12	2.19	6.31	65.3	34.7
6	21.79	14.63	36.42	59.8	40.2
Mean	5.63	3.65	9.28	61.3	38.7
S.D.	7.99	5.40	13.39	5.47	5.47

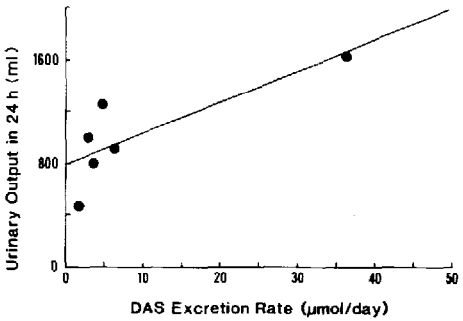

Fig. 3. Correlation between the daily urine volume and total DAS urinary excretion rate in humans ($r = 0.800$, $p < 0.001$).

There was a significant positive correlation ($r = 0.800$, $p < 0.001$) between DAS excretion rate and the volume of urine excreted in a 24-h period (Fig. 3). There was a weaker correlation ($r = 0.603$, $0.05 < p < 0.10$) when the highest data point was dropped from the analysis. These correlations are interesting since it has recently been shown that DA-3-S inhibits the angiotensin-stimulated release of aldosterone in cultured bovine adrenal cells [7]. Those authors postulated that DA-3-S might complement the dopaminergic inhibition of aldosterone secretion by the adrenal gland. Our findings support this hypothesis since inhibition of aldosterone would have the effect of increasing urinary volume.

CONCLUSIONS

This paper describes an accurate, reproducible and relatively simple sample preparation procedure for measuring DA-3-S and DA-4-S in human urine. By using SPE columns, DAS isomers can be routinely quantified in urine samples by HPLC with dual-electrode electrochemical detection. The readiness of this procedure for measuring urinary DAS isomers should aid in the direct examination of their physiological and metabolic roles.

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