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DIRECT MEASUREMENT OF DOPAMINE O-SULFATE IN PLASMA AND CEREBROSPINAL FLUID

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SUMMARY

This paper describes a method for measurement of dopamine 3-O-sulfate (DA3S) and dopamine 4-O-sulfate (DA4S) in dog and human plasma and dog cerebrospinal fluid. C_{18} solid-phase extraction columns were utilized for sample preparation. DA3S and DA4S were separated by high-performance liquid chromatography and then quantified by dual-electrode electrochemical detection. [^{3}H]Dopamine O-sulfate (DAS) was used as internal standard. Recovery of authentic DAS added to dog plasma and carried through the entire procedure was 49.9 ± 6.3% for DA3S ($n = 9$) and 42.2 ± 4.3% for DA4S ($n = 8$). The lower limit of detection (signal-to-noise ratio of 3) was 20 fmol for each DAS isomer. The within-assay coefficient of variation for DA3S in dog plasma averaged 5.8% (range 2.0—12%, $n = 5$). The between-assay coefficient of variation for DA3S in dog plasma was 3.8% ($n = 3$). DAS levels in plasma of conscious dogs were 20.3 ± 6.9 pmol/ml DA3S and 5.91 ± 3.5 pmol/ml DA4S ($n = 5$). Cerebrospinal fluid levels were 3.06 ± 3.22 pmol/ml DA3S and 0.10 ± 0.18 pmol/ml DA4S in dogs anesthetized with methoxyflurane and nitrous oxide ($n = 3$). This procedure is also appropriate for use with human plasma; DAS levels were 24.3 ± 12.8 pmol/ml DA3S and 9.07 ± 3.9 pmol/ml DA4S ($n = 6$).

INTRODUCTION

It has been established that more than 95% of dopamine (DA) in human plasma is present in the sulfated form [1—3]. However, no simple method for direct measurement of plasma dopamine O-sulfate (DAS) has been reported. Most procedures for estimation of DAS levels involve hydrolysis of the sulfate group and report conjugated DA as the difference between total (hydrolyzed) DA and free (unhydrolyzed) DA levels in single or duplicate aliquots. These

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procedures result in loss of the distinction between the two isomers of DAS, dopamine 3-O-sulfate (DA3S) and dopamine 4-O-sulfate (DA4S), when enzymatic (sulfatase) hydrolysis is used and loss of the distinction between DAS and other conjugates of DA when alkaline or acid hydrolysis is used. This paper describes a simple method for direct measurement of DA3S and DA4S in human and dog plasma and dog cerebrospinal fluid (CSF).

EXPERIMENTAL

Chemicals

Chemicals for all experiments were analytical or reagent grade. Dopamine hydrochloride and deoxyepinephrine hydrobromide were purchased from Sigma (St. Louis, MO, U.S.A.). [8-^{3}H]Dopamine (20—30 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Dopamine 3-O-sulfate, dopamine 4-O-sulfate, dopamine 4-O-glucuronide, serotonin O-sulfate, norepinephrine 3-O-sulfate, norepinephrine 4-O-sulfate, 3-methoxy-4-(sulfonyloxyphenyl)glycol (MHPG sulfate), tyramine O-sulfate and 3-methoxy-4-(hydroxysulfonyloxy)phenylacetic acid (homovanillic acid sulfate) were obtained from Dr. J.S. Kennedy (NIMH, Bethesda, MD, U.S.A.).

Apparatus

The high-performance liquid chromatographic (HPLC) systems utilized either a 6000A or M45 pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne 7125 sample injection valve equipped with a 100-μl loop (BAS, West Lafayette, IN, U.S.A.), and a Houston Instruments strip-chart recorder (BAS). Each system was equipped with a guard column (2 cm × 4.5 mm) packed with 40-μm C_{18} Corasil (Waters Assoc.) and a stainless-steel analytical column (25 cm × 4 mm) prepacked with 5-μm octadecyl silica (Biophase ODS, BAS). Three different detectors were utilized: (1) a dual-electrode electrochemical detector (LC-4B-17D, BAS) consisting of a thin-layer dual glassy-carbon working electrode (modified for fraction collection) used in the series configuration, an Ag/AgCl reference electrode, and two LC-4B amperometric controllers; (2) an ultraviolet (UV) detector (Model 440, Waters) equipped with a 280-nm filter; and (3) a flow-through scintillation counter (Flo-One, Radiomatic Instruments, Tampa, FL, U.S.A.).

Sample collection and deproteination

All samples were obtained from mature males and collected in EDTA-containing vacuum tubes (Vacutainer, Becton-Dickinson, Rutherford, NJ, U.S.A.) on ice. Blood was drawn from humans (ages 20—35 years) via the antecubital vein and from conscious mixed breed dogs via the jugular or cephalic vein. In addition, blood and CSF were obtained from healthy mixed breed male dogs anesthetized with methoxyflurane and nitrous oxide; blood was drawn from the femoral vein and free-flowing CSF was collected from the foramen magnum. CSF samples with significant red blood cell (RBC) contamination (>1000 RBC/mm^3) were discarded. The blood samples were centrifuged at 3900 *g* at 4°C for 20 min and the plasma and CSF stored at −70°C until analysis. All human subjects gave written informed consent prior to blood

collection, and the protocol was approved by the Purdue University Human Research Committee.

For the analysis of DAS, samples were thawed at room temperature, diluted with doubly distilled water (two volumes for plasma, one volume for CSF), internal standard added, and 4 *M* perchloric acid (PCA) added to produce a final concentration of 0.4 *M* PCA. Samples were centrifuged for 20 min at approximately 12 000 *g* at 4°C, the protein pellet was discarded, and the deproteinated sample assayed for DAS.

Sample preparation

Samples were prepared by a modification of the method of Swann and Elchisak [4]. The deproteinated supernatants were transferred to new tubes, and 3 *M* Tris (approximately 350 μl per 1 ml plasma) was added to adjust the pH to 7—7.5. The samples were then subjected to the sample preparation procedure utilizing C_{18} solid-phase extraction (SPE) columns shown in Fig. 1. The SPE columns (Bond-Elut, 3 ml capacity, containing 500 mg octadecyl silica, Analytichem, Harbor City, CA, U.S.A.) were conditioned immediately before use by washing with two column volumes of methanol and one column volume of 1% EDTA using a Baker 10 SPE system (J.T. Baker, Phillipsburg, NJ, U.S.A.). A vacuum of approximately 15 mmHg was applied to aspirate all solutions to the top of the sorbent level. The sample was applied to the column and aspirated. The column was washed with two 0.5-ml aliquots of 0.5 *M* acetic acid, and the sample was then eluted with two 0.5-ml aliquots of 0.5 *M* acetic acid. The eluate was kept on ice until all samples were processed and was then evaporated to dryness in a vacuum centrifuge at approximately 550 *g* (Speed-Vac concentrator, Savant, Hicksville, NY, U.S.A.). The samples were dissolved in an appropriate volume of the mobile phase used for the HPLC analysis of the samples.

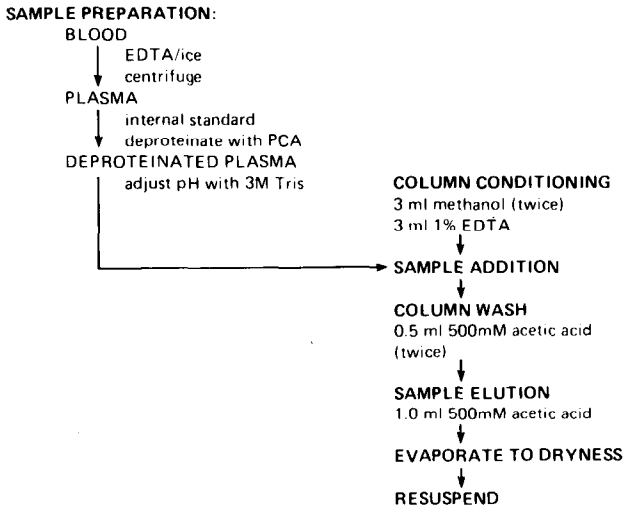

Fig. 1. Sample preparation procedure for measurement of DA3S and DA4S in plasma and CSF using solid-phase extraction columns. Disposable 3-ml columns, packed with octadecylsilica (40 μm), were used. DAS was then quantitated by dual-series electrode electrochemical detection after HPLC.

Recovery of DAS from dog plasma was determined after HPLC by comparison of peak heights from plasma, with and without addition of authentic DAS, carried through the sample preparation procedure to peak heights of known amounts of authentic DAS injected directly into the HPLC system.

The SPE columns were cleaned after use by sequential rinsing with one column volume of each of the following solvents: methanol, chloroform, hexane, chloroform, methanol, 0.5 *M* acetic acid, 2 *M* potassium chloride, distilled water, and 0.5 *M* acetic acid. Columns were used for plasma samples at least eight times without a noticeable decrease in recovery of the DAS isomers. Repeated uses of the SPE columns were eventually limited by clogging of the frits; there was no noticeable decrease in column efficiency.

Internal standard preparation

^{3}H[DAS] was used as internal standard. [^{3}H]DAS was synthesized from [^{3}H]DA (specific activity 20—30 Ci/mmol) by a modification of the method of Jenner and Rose [5]. All procedures were conducted with pre-chilled solvents and the reaction vessel was kept on ice. A 40-μl volume of [^{3}H]DA (1 mCi/ml) was evaporated to dryness under a stream of nitrogen, resuspended in 40 μl of absolute ethanol, and dried again. Sulfuric acid (20 μl) was added to the dried [^{3}H]DA and the mixture was stirred for 20 min. A 200-μl volume of doubly distilled cold water was then added and the mixture stirred briefly.

The crude synthetic mixture was initially purified by HPLC and collection of the fraction that contained the [^{3}H]DAS isomers (based on retention times of the authentic DAS isomers). The mobile phase was 2% (v/v) methanol in water [6]. UV detection at 280 nm was utilized. Typical capacity factors were 4.2 and 4.5 for DA4S and DA3S, respectively. Free DA was indefinitely retained on the column. The purity of the [^{3}H]DAS fraction collected was determined using another HPLC system. The mobile phase was 75 m*M* KH_2PO_4 and 1 m*M* EDTA, pH 4.8, and a UV detector and a flow-through scintillation counter were utilized in series. Since authentic [^{3}H]DAS isomers were not available, retention times of authentic non-radioactive DAS isomers were determined by UV detection and converted to appropriate retention times for the flow-through scintillation counter. The purified [^{3}H]DAS mixture was utilized for internal standard only if greater than 90% of the counts were due to DAS. The [^{3}H]DAS solution was then diluted with doubly distilled water such that it contained approximately 2500 cpm per 50 μl and stored in 500-μl aliquots at −70°C.

Internal standard utilization

When the processed sample was injected into the HPLC system used for routine analyses (described below), the fraction of column eluate containing the DAS isomers was collected, added to 13.5 ml of Flo-Scint III scintillation cocktail (Radiomatic Instruments), and the [^{3}H]DAS counted with a Beckman LS-7500 scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.). The amount of DAS in each sample was calculated by linear regression analysis of a standard curve constructed by addition of internal standard to known amounts of each DAS isomer which were then carried through the sample preparation and HPLC procedures. The amount of [^{3}H]DAS used as internal

standard was below the lower limits of detection of the system and did not contribute to the measured DAS content of the samples.

HPLC conditions

Amounts of DA3S and DA4S were determined in plasma samples by HPLC with dual-series electrode electrochemical detection. The upstream working electrode was maintained at + 1.00 V and the downstream electrode at 0.00 V versus the reference electrode. The mobile phase was 75 m*M* phosphate buffer (pH 4.8) containing 1 m*M* EDTA [7]. The flow-rate was isocratic at 0.8—1.2 ml/min and produced a pressure of approximately 80—140 bar.

RESULTS AND DISCUSSION

DAS assay

Typical chromatograms of DA3S and DA4S in standards, plasma, and CSF carried through the sample preparation procedure (shown in Fig. 1) are shown in Fig. 2. The relationship between the amount of DA3S and DA4S injected into the HPLC system and the ratio of peak height to [^{3}H]DAS, the internal standard (cpm) was linear from 0.2 to 50 pmol. The lower limit of detection was approximately 20 fmol for each of the DAS isomers at a signal-to-noise ratio of 3.

Recovery of authentic DAS added to dog plasma and carried through the entire procedure was 49.9 ± 6.3% for DA3S (n = 9) and 42.2 ± 4.3% for DA4S (n = 8). The within-assay coefficient of variation (C.V.) for DAS standards (100 pmol) carried through the sample preparation and HPLC procedures was 7.1—9.7% for DA3S and 3.8—6.6% for DA4S in four assays. The within-assay

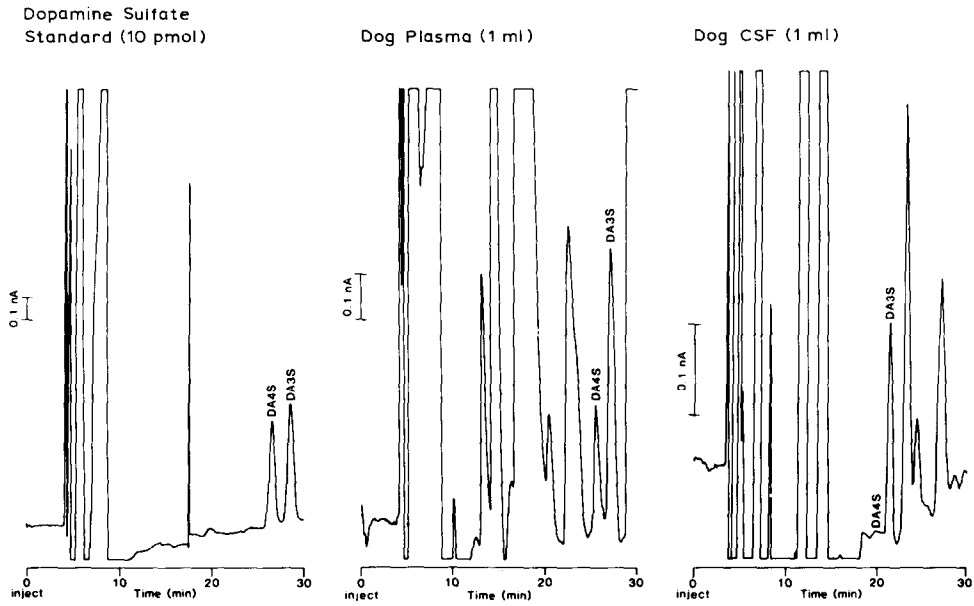

Fig. 2. HPLC profiles of DAS in standard solutions, dog plasma, and dog CSF after the sample preparation procedure shown in Fig. 1. Retention times of the DAS differ because assays from different days are shown.

C.V. for DA3S in dog plasma averaged 5.8% (range 2.0—12%, $n = 5$). The between-assay C.V. for DA3S in dog plasma was 3.8% ($n = 3$).

Assay of other compounds

A series of compounds that might interfere with the measurement of DA3S and DA4S in this assay, or be of interest themselves, was injected into the HPLC system. Capacity factors were calculated, and these are shown in Table I.

TABLE I

CAPACITY FACTORS FOR CATECHOL-LIKE COMPOUNDS

Capacity factor = [(peak time − void volume time)/void volume time].

Compound	Capacity factor	Compound	Capacity factor
Dihydroxymandelic acid	0.37	N-Acetyldopamine	4.00
Norepinephrine	0.48	Dopamine 4-O-sulfate	4.73
Caffeic acid	0.49	Dopamine 3-O-sulfate	5.18
L-3,4-Dihydroxyphenylalanine	1.23	Deoxyepinephrine	5.41
Epinephrine	1.53	Tyramine	6.11
Dihydroxybenzylamine	1.59	3,4-Dihydroxyphenylacetic acid	7.98
Homovanillic acid	1.88	3-Methoxy-4-hydroxyphenylglycol	8.58
Tyramine O-sulfate	2.14	Homovanillic acid O-sulfate	11.76
Dihydroxyphenylglycol	2.43	Isoproterenol	12.59
Dopamine 4-O-glucuronide	2.50	Serotonin	15.13
α-Methyldihydroxyphenylalanine	2.84	Serotonin O-sulfate	15.93
α-Methyldopamine	2.88	Dihydrocaffeic acid	22.06
Uric acid	3.00	3-Methoxy-4-hydroxyphenylglycol sulfate	34.38
Dopamine	3.34		
Norepinephrine 4-O-sulfate	3.86		
Norepinephrine 3-O-sulfate	3.93		

DAS in dog plasma and CSF

In plasma obtained from conscious dogs ($n = 5$), the concentrations of DA3S and DA4S were 20.3 ± 6.9 and 5.91 ± 3.5 pmol/ml, respectively. CSF levels were 3.06 ± 3.2 pmol/ml DA3S and 0.10 ± 0.18 pmol/ml DA4S in dogs anesthetized with methoxyflurane and nitrous oxide ($n = 3$). To the best of our knowledge, these are the first reported values for the separate isomers of DAS in canine plasma. There are few reported values for conjugated DA in dog plasma. Our value for total DAS in dog plasma, 26.6 ± 6.9 pmol/ml, is somewhat greater than the conjugated DA levels of approximately 10 pmol/ml reported by Unger et al. [8] and Bove et al. [9]. It is difficult to reconcile these differences, even though very different techniques were utilized. However, we have observed large variations in DAS levels in plasma drawn from dogs exposed to different amounts of external stimulation. In anesthetized dogs ($n = 3$; anesthetized with methoxyflurane and nitrous oxide), we found plasma levels of 6.5 pmol/ml DAS (5.35 ± 1.2 pmol/ml DA3S and 1.14 ± 0.37 pmol/ml DA4S. We have observed plasma DAS concentrations up to 40 times higher than these in dogs which were subjected to various types of stress, such

as difficult venipuncture, excessive heat, presence of other animals, etc. These data suggest that DAS levels in plasma may be sensitive to environmental input and emphasize the need for uniform sampling procedures.

DAS in human plasma

Since the presence and concentrations of DAS have been well characterized in human plasma, but not in dog plasma, we utilized human plasma to further validate our procedure. DAS levels in the plasma of conscious males ($n = 6$) were 24.3 ± 12.8 and 9.07 ± 3.9 pmol/ml for DA3S and DA4S, respectively. These values agree well with those recently reported by Yamamoto et al. [10]. The sum of our values for DA3S and DA4S in human plasma are also comparable to values for total DAS (DA3S + DA4S) reported by other investigators [2, 8, 11—14].

The procedure reported here should prove very useful for studies concerning DAS in human plasma. Although another procedure has recently been reported, our sample preparation method is more rapid and our detection method more sensitive and less tedious to utilize than the other procedure [10].

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REFERENCES

1 N.T. Buu and O. Kuchel, J. Lab. Clin. Med., 90 (1977) 680.
2 G.A. Johnson, C.A. Baker and R.T. Smith, Life Sci., 26 (1980) 1591.
3 G.R. Van Loon, Fed. Proc., Fed. Am. Soc. Exp. Biol., 42 (1983) 3012.
4 P.G. Swann and M.A. Elchisak, J. Chromatogr., 381 (1986) 241.
5 W.N. Jenner and F.A. Rose, Nature, 252 (1974) 237.
6 B.A. Osikowska, J.R. Idle, F.J. Swinbourne and P.S. Sever, Biochem. Pharmacol., 31 (1982) 2279.
7 N.T. Buu, G. Nair, O. Kuchel and J. Genest, J. Lab. Clin. Med., 98 (1981) 527.
8 T. Unger, N.T. Buu, O. Kuchel and W. Schurch, Can. J. Physiol. Pharmacol., 58 (1980) 22.
9 A.A. Bove, J.D. Dewey and G.M. Tyce, J. Lab. Clin. Med., 104 (1984) 77.
10 T. Yamamoto, A. Yamatodani, M. Nishimura and H. Wada, J. Chromatogr., 342 (1985) 261.
11 L.A. Toth, M.C. Scott and M.A. Elchisak, Life Sci., 39 (1986) 519.
12 J.L. Cuche, J. Prinseau, F. Selz, G. Ruget, J.L. Tual, L. Reingeissen, M. Devoisin, A. Baglin, J. Guedon and D. Fritel, Hypertension, 7 (1985) 81.
13 O. Kuchel, N.T. Buu, P. Hamet and P. Larochelle, Clin. Pharmacol. Ther., 37 (1985) 372.
14 O. Kuchel, C. Hausser, N.T. Buu and S. Tenneson, J. Neural Transm., 62 (1985) 91.