Journal of Chromatography, 342 (1985) 261—267
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2648

DETERMINATION OF DOPAMINE-3- AND -4-O-SULPHATE IN HUMAN PLASMA AND URINE BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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(First received February 8th, 1985; revised manuscript received March 13th, 1985)

SUMMARY

A high-performance liquid chromatographic (HPLC) method is reported for the determination of dopamine-3- and -4-O-sulphate isomers in human plasma and urine using an anion exchanger coupled with post-column hydrolysis and fluorimetric detection. Samples of plasma or urine are partially purified on Dowex 1 and Dowex 50 columns and separated using HPLC. These compounds are then hydrolysed and determined automatically by the *p*-aminobenzoic acid method in a continuous-flow reaction system. As the *p*-aminobenzoic acid method is very specific for dopamine, it is also possible to determine the isomers by injecting 5—20 μl of urine or 100—200 μl of deproteinized plasma directly into the HPLC system without clean-up.

The detection limit of the method for both isomers is 0.3 pmol. In normal subjects, the plasma levels of dopamine-3- and -4-O-sulphate are 26.5 (S.D. 11.1) and 2.68 (S.D. 0.34) pmol/ml, and their urinary excretion rates are 1.73 (S.D. 0.56) and 0.27 (S.D. 0.04) nmol/min, respectively. Thus the two isomers are present in both plasma and urine and their urinary excretions reflect directly their plasma levels.

0378-4347/85/$03.30

INTRODUCTION

Circulating dopamine is mainly present in conjugated forms, and its sulphate esters are the predominant conjugates in human plasma [1—3]. It is still controversial whether these dopamine conjugates are simple inactivated products of dopamine, whether they are intermediates in the biosynthesis of catecholamines [4, 5] or whether they themselves have physiological activity [6, 7]. The examination of their possible roles requires the use of a sensitive and specific method. Although the sulpho-conjugated dopamines have been determined by enzymatic hydrolysis [1—3], it is impossible to distinguish the two isomers by this method. Recently, Arakawa et al. [8] and Elchisak [9] reported a high-performance liquid chromatographic (HPLC) separation of the two isomers and demonstrated the presence of both the 3- and 4-O-sulphates in human urine using a photochemical and electrochemical detector coupled with post-column hydrolysis, respectively. However, a more sensitive and specific method is required for the determination of their plasma levels.

Hamaji and Seki [10] reported a highly sensitive and specific method for the fluorimetric detection of free dopamine in plasma. By combination of this fluorimetric method with anion-exchange HPLC and post-column hydrolysis, we have developed a direct assay that is sufficiently sensitive for the measurement of the plasma levels of dopamine sulpho-conjugated isomers. Using this method, we have demonstrated the presence of both dopamine-3- and -4-O-sulphate in plasma and urine from normal human subjects.

EXPERIMENTAL

Dopamine-3- and -4-O-sulphate

Dopamine-3- and -4-O-sulphate were synthesized by the method of Jenner and Rose [11] with some modifications as follows.

Dopamine hydrochloride (0.4 g) (Sigma, St. Louis, MO, U.S.A.) was added with stirring during 20 min to 1.1 ml of sulphuric acid (analytical-reagent grade) (Wako, Osaka, Japan) in a small test-tube in an ice-bath. The reaction mixture was then quickly poured into 10 ml of ice-cold water with vigorous stirring. The diluted solution was promptly applied to a Dowex 50-X8 column (40 × 1.2 cm I.D., 200—400 mesh, H^+) and eluted with distilled water. The absorbance of the eluate was monitored spectrophotometrically at 280 nm. After discarding about 40 ml of the eluate, the sulphated dopamines were eluted in a volume of about 30 ml of eluate. This fraction was reduced to 10 ml in vacuo at 30°C and then applied to a Dowex 1-X2 column (30 × 2.0 cm I.D., 200—400 mesh, acetate form) and eluted with 0.2 *M* acetic acid. A 20-μl portion of each fraction (9 ml) of the eluate was analysed by reversed-phase HPLC [9] with a UV detector to determine the elution profiles of the two isomers. Fig. 1 shows typical elution patterns of the 3- and 4-O-sulphates on a Dowex 1 column. Fractions corresponding to the 3- and 4-O-isomers were evaporated to dryness and then recrystallized from hot ethanol—water (4:1, v/v) and 0.05 *M* acetic acid, respectively. Structural assignment of the final products was achieved by high-resolution ^1H NMR spectroscopy (Nicolet Model NT-360) [12]. The purity of the final products was over 95%, as judged by HPLC and NMR spectrometry.

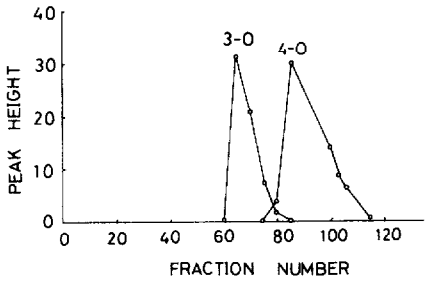

Fig. 1. Separation and purification of chemically synthesized dopamine sulphates by Dowex 1 column chromatography. Partially purified sulphated dopamines from a Dowex 50-X8 column were applied to a Dowex 1-X2 column (30 × 2.0 cm I.D.). A portion of each 9-ml fraction was injected into a reversed-phase HPLC column coupled with a UV detector, and the absorbance at 280 nm was monitored. The peak heights corresponding to dopamine-3-(3-O) and -4-O-sulphate (4-O) on the chromatogram were plotted against the fraction number. The isomers were identified by ^{1}H NMR analysis of the final purified preparations.

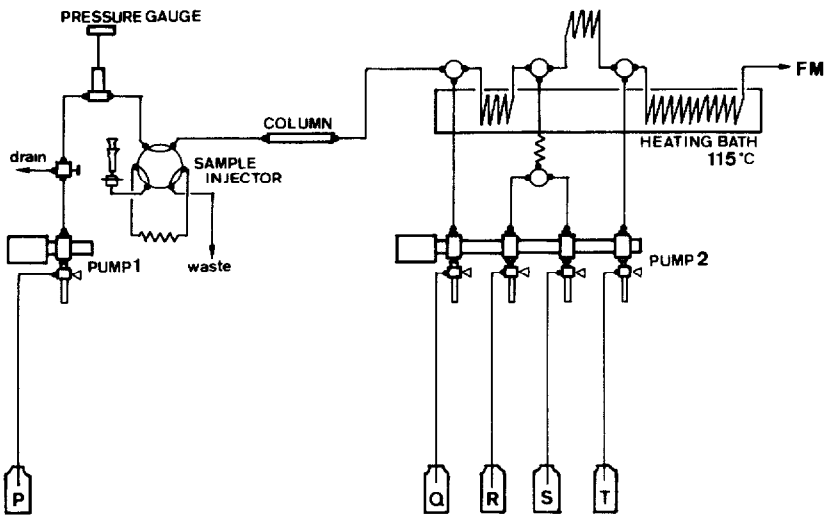

Fig. 2. Flow diagram of the HPLC system. Column: TSK-gel DEAE2SW (5 μm, 25 cm × 4.6 mm I.D.). Mobile phase: (P) 25 m*M* sodium phosphate buffer, pH 6.5, 0.5 ml/min. Reagents: (Q) 0.92 *M* perchloric acid, 0.15 ml/min; (R) 0.6% *p*-aminobenzoic acid, pH 6.3, 0.20 ml/min; (S) 1.3 *M* sodium hydroxide—0.4 *M* boric acid mixture, 0.20 ml/min; (T) 0.7% potassium hexacyanoferrate(III), pH 9.3, 0.20 ml/min. FM = Fluoromonitor. The excitation and emission wavelengths were 475 and 515 nm, respectively.

Chromatographic apparatus and conditions

Fig. 2 shows a schematic diagram of the chromatographic system. The mobile phase (25 m*M* sodium phosphate buffer, pH 6.5) was delivered at 0.5 ml/min using a constant-flow pump (pump 1) (LDC Constametric II; ATTO, Tokyo, Japan). The sample injector was a Rheodyne Model 7125 valve; the stainless-steel column was pre-packed with an anion exchanger (TSK-gel DEAE2SW, 5 μm, 25 cm × 4.6 mm I.D.; Toyo Soda, Tokyo, Japan). The plungers of a quadruple plunger pump (pump 2 in Fig. 2) (Model SF-4; Toyo Soda) served to deliver reagents Q, R, S and T independently. The column

eluate was first mixed with 0.92 *M* perchloric acid (Q, 0.15 ml/min) through polytetrafluoroethylene tubing (10 m × 0.5 mm I.D.) at 115°C in a closed water-bath. To the acid-hydrolysed eluate, 0.6% (w/v) *p*-aminobenzoic acid solution (R, 0.20 ml/min, adjusted to pH 6.3 with 6 *M* sodium hydroxide) and 1.3 *M* sodium hydroxide containing 0.4 *M* boric acid (S, 0.20 ml/min) were added with mixing in tubing (2 m × 0.5 mm I.D.). Finally, 0.7% (w/v) potassium hexacyanoferrate(III) solution (T, 0.20 ml/min, adjusted to pH 9.3 with 6 *M* sodium hydroxide) was added with mixing in a long reaction tube (20 m × 0.5 mm) at 115°C to develop the fluorophore. The pH of the final reaction mixture was 9.3.

The fluorescence intensity was measured at 515 nm using an excitation wavelength of 475 nm in a spectrofluorimeter (Model 650-10LC; Hitachi Seisakusho, Tokyo, Japan) equipped with a 90-μl square flow cell and a recorder (Model 056; Hitachi Seisakusho).

Sample preparation and clean-up

Blood was drawn from the antecubital vein of healthy adults into a heparinized syringe at the mid-point during collection of a 1-h urine sample. The blood was promptly transferred into an ice-chilled centrifuge tube containing a mixture of 0.2 *M* disodium EDTA and 0.2 *M* $Na_2S_2O_5$ (10 μl/ml of blood; adjusted to pH 6—7 with 6 *M* sodium hydroxide). After gentle mixing by inversion of the tube, plasma was separated by centrifugation at 4000 *g* for 10 min at 4°C. To 1.0 ml of the plasma, 0.5 ml of 2.5% perchloric acid was added and the mixture was stored at −80°C until taken for analysis. Under these conditions the two isomers were stable for at least one month. The mixture was centrifuged at 10 000 *g* for 30 min at 4°C and then the deproteinized supernatant (1.0 ml) was adjusted to pH 7.0 by dropwise addition of a mixture of 2 *M* potassium hydroxide and 1 *M* potassium dihydrogen phosphate. Insoluble potassium perchlorate was removed by brief centrifugation, and the supernatant was applied to a small column of Dowex 1-X2 (20 × 4 mm I.D., 200—400 mesh, acetate form). The column was washed with 2.0 ml of distilled water and then material was eluted with 4.0 ml of 0.5 *M* acetic acid. The eluate was introduced directly into a second column of Dowex 50-X8 (20 × 4 mm I.D., 200—400 mesh, H^+) and the flow-through fraction was collected and evaporated to dryness. The residue was dissolved in 250 μl of distilled water and 50—200 μl of the solution were injected into the HPLC system.

The procedure for partial purification of urine samples (1.0 ml) was the same as that for plasma, except that deproteinization and final evaporation were not required, and 100 μl of the eluate from the Dowex 50 column were injected directly into the HPLC system.

The recoveries of the partial purification procedure were determined from the peak heights of samples spiked with known amounts of the two isomers and subjected to the same procedure.

RESULTS AND DISCUSSION

Figs. 3 and 4 show typical chromatograms of dopamine-3- and -4-O-sulphate

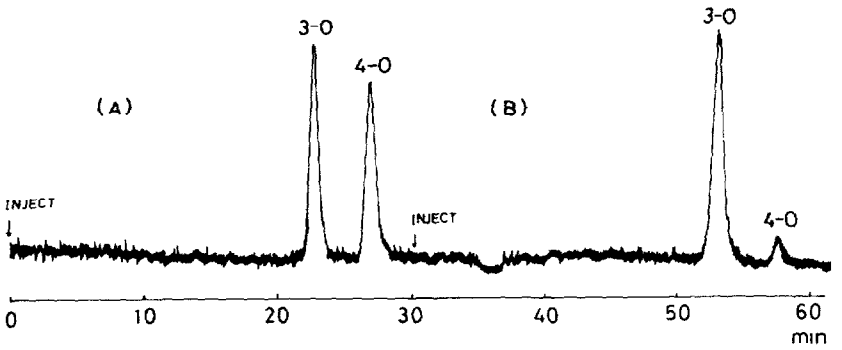

Fig. 3. Chromatograms of standard and plasma samples. (A) Elution pattern of the standard mixture containing 2.5 pmol each of dopamine-3- (3-O) and -4-O-sulphate (4-O). (B) Elution pattern of a plasma sample. The volume of sample injected corresponds to 133.3 μl of plasma and the plasma concentrations corrected for the recovery rates are 24.9 pmol/ml for 3-O and 2.9 pmol/ml for 4-O, respectively.

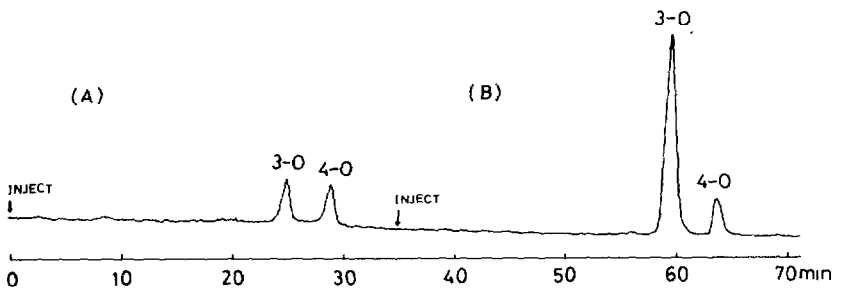

Fig. 4. Chromatograms of standard and urine samples. (A) Elution pattern of the standard mixture containing 5 pmol each of dopamine-3- (3-O) and -4-O-sulphate (4-O). (B) Elution pattern of a urine sample. The amount of sample injected corresponds to 20 μl of urine and the urinary concentrations corrected by the recovery rates are 4.46 nmol/ml for 3-O and 0.86 nmol/ml for 4-O, respectively.

in normal human plasma and urine. The two isomers were well separated and no interfering peaks appeared on the chromatogram. As the fluorimetric detection method using *p*-aminobenzoic acid is very specific for dopamine [7], it is also possible to determine the isomers by injecting 10 μl of the urine or 100—200 μl of the deproteinized plasma directly into the HPLC system without any purification procedures (Fig. 5), although with this procedure the column lifetime is decreased.

The amounts of the two isomers injected in the range 1—20 pmol are linearly related to the fluorescence intensities expressed as peak heights. The linear regression equations and correlation coefficients for dopamine-3- and -4-O-sulphate were $y = 0.379x + 0.129$ ($r = 0.99962$) and $y = 0.368x - 0.004$ ($r = 0.99983$), respectively. The detection limit (signal-to-noise ratio = 2) was 0.3 pmol for each isomer.

When 12.5 pmol of each isomer were added to 1.0 ml of pooled plasma, the overall recoveries were 78.3 ± 3.2% for dopamine-3-O-sulphate and 79.9 ± 1.3% for dopamine-4-O-sulphate ($n = 5$). The between-day (days 1, 2, 3, 7 and 14) coefficient of variation was 9.6% for dopamine-3-O-sulphate and 5.0%

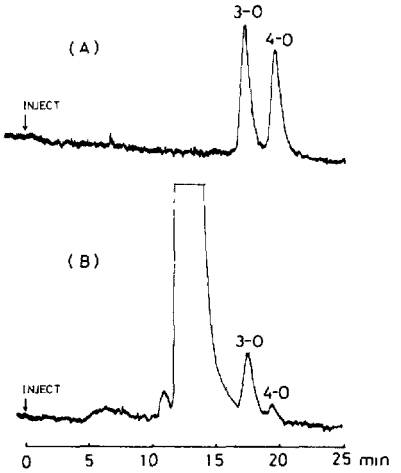

Fig. 5. Chromatogram of plasma sample injected directly into the HPLC column without any clean-up. (A) Elution pattern of the standard mixture containing 5 pmol each of dopamine-3- (3-O) and -4-O-sulphate (4-O). (B) Elution pattern of 200 μl of the deproteinized plasma injected directly into the HPLC column. The plasma concentrations for 3-O and 4-O are 21.8 and 4.4 pmol/ml, respectively.

TABLE I

PLASMA LEVELS AND URINARY EXCRETION RATES OF DOPAMINE-3- AND 4-O-SULPHATE IN NORMAL HUMAN SUBJECTS

Values are means ± S.D. (n = 10 for plasma; n = 5 for urine).

Dopamine sulphate	Plasma level (pmol/ml)	Urinary excretion rate (nmol/min)
3-O-Sulphate	26.5 ± 11.1	1.73 ± 0.56
4-O-Sulphate	2.68 ± 0.34	0.27 ± 0.04

for dopamine-4-O-sulphate, and the within-day coefficient of variation was less than 5% for both isomers (n = 5).

As shown in Table I, the mean concentrations of dopamine-3- and -4-O-sulphate in normal human plasma were 26.5 ± 11.1 and 2.68 ± 0.34 pmol/ml (n = 10), respectively. The mean urinary excretion rates in normal subjects were 1.73 ± 0.56 for the 3-O-sulphate and 0.27 ± 0.04 nmol/min for the 4-O-sulphate. These data are in good agreement with those determined by enzymatic hydrolysis [1—3] (plasma levels) and with an HPLC—photochemical detector [8] (urine levels).

The ratios of dopamine-3- to -4-O-sulphate in the plasma and urine obtained in this work were almost the same. Further, the clearance rates of the two isomers calculated from the present data (85—100 ml/min) are nearly equal to that of creatinine. These results suggest that the urinary levels of dopamine sulphates directly reflect the plasma concentrations of the isomers in man.

ACKNOWLEDGEMENTS

We thank Dr. Yoko Naya, Vice Director of Suntory Institute for Bioorganic

Research for facilitating the ^{1}H NMR analysis of the isomers, Ms. K. Tsuji for typing the manuscript and Mr. T. Ishii for technical assistance.

This work was partly supported by grants from the Ministry of Health and Welfare of Japan (Cardiovascular Disease 56A-7), and from the Descente Foundation for the Promotion of Sports Science.

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