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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DOPAMINE SULFOCONJUGATES IN URINE AFTER L-DOPA ADMINISTRATION*

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SUMMARY

A procedure was developed for the separation and determination of dopamine-3-O-sulfate (DM-3-S) and dopamine-4-O-sulfate (DM-4-S) in the urine of subjects administered L-DOPA. The method consists of sample preparation using cation- and anion-exchange resins followed by determination of the sulfates by high-performance liquid chromatography. The addition recoveries were 96 ± 2.9% (S.D.) for DM-3-S and 93 ± 3.0% (S.D.) for DM-4-S. Twenty samples could be measured per day. When every 2-h urine specimen from normal subjects was analysed after L-DOPA administration (0.5 g), the maximum excretion of each sulfate was observed in the second 2-h specimen. For the first 6 h 7.5 ± 1.5% (S.D.) of the administered L-DOPA was excreted as DM-O-sulfates. During this time, the ratio of DM-4-S to the DM-O-sulfates was 11.7 ± 0.58% (S.D.).

INTRODUCTION

From the results of acid and enzyme hydrolysis, the main conjugate of dopamine (DM) in urine had been thought to be O-sulfate [1—5]. Jenner and Rose [6] first identified the conjugates as DM-3-O-sulfate (DM-3-S) and DM-4-O-sulfate (DM-4-S), which were excreted in the urine of Parkinsonian patients with the intake of high doses of L-DOPA, an immediate precursor of DM. They also reported that a rat brain preparation sulfated DM to DM-3-S and DM-4-S in the ratio 1.7:1, whereas rat liver supernatant produced mainly DM-3-S, suggesting the importance of separate determination of these conjugates [7]. The method they used for the estimation of the sulfates in urine was time-consuming and tedious. Recently, Bronaugh et al. [8] developed a method to

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estimate the conjugates by high-performance liquid chromatography (HPLC). However, the sample preparation procedure they used required more than 12 h and the separation of DM-3-S from DM-4-S by HPLC seemed incomplete even with a 2-m column of Zipax SAX (DuPont), and the sensitivity of the detection was low.

In this paper we report on a simpler, more rapid and sensitive method for the measurement of DM-3-S and DM-4-S in urine. The method consists of isolating the conjugates from urine by passing the urine successively through small columns of cation and anion exchange resin, and the quantitation of the conjugates by using silica gel HPLC to separate them. The method was applied to the estimation of the conjugates in the urine of normal subjects who were administered 0.5 g of L-DOPA orally.

EXPERIMENTAL

Materials

Dowex 50W-X8 (200—400 mesh; Dow Chem., Midland, Mich., U.S.A.) and Dowex 1-X8 (200—400 mesh) were used. DM-3-S and DM-4-S were synthesized according to the method of Jenner and Rose [7]. Wakogel LC-5K (spherical silica gel for HPLC, 5 μm, Lot No. LDM4300) was the gift from Wako (Osaka, Japan). All the other chemicals were of reagent grade.

Administration of L-DOPA and collection of urine

Subjects were 9 normal men (from 22 to 35 years; the mean age was 25.8 years). The subjects were obliged to fast after the last evening meal until 1 p.m. The first morning urine was discarded. Then some water was taken, and at 9 a.m. the first urine (0-h urine) was collected and 0.5 g of L-DOPA (Larodopa: Hoffman-La Roche) was administered orally. 2, 4 and 6 h later, every 2-h urine was collected and designated 0—2 h, 2—4 and 4—6 urine, respectively. To each sample were added 50 mg of ascorbic acid and 25 mg of EDTA-2Na to preserve catecholamines. The urines were stored at —20°.

Sample preparation for HPLC

The urine sample was thawed and centrifuged at 1000 *g* for 5 min. One ml of the supernatant was applied to a Dowex 50W (H+) column (6.4 cm × 1.0 cm I.D.) in a cold room at 6°, and the column was washed with 4 ml of chilled water. The effluent and the washings were discarded. Then a column (1.1 cm × 0.5 cm I.D.) of Dowex 1 (Cl^-) was connected to the bottom of the Dowex 50W column. A 25-ml volume of chilled water was added to the top of the Dowex 50W column to elute the DM-O-sulfates from both the columns. The eluate from the Dowex 1 column was rotary evaporated under a reduced pressure by a vacuum pump at 30°. The resultant residue was redissolved in 100 μl of water and transferred to a conical tube (Eppendorf Reactionsgefaesse 3810, 4 cm × 0.9 cm I.D.). The tube was centrifuged at 1300 *g* for 3 min. Five μl of the supernatant were analysed by HPLC.

HPLC of the conjugates

Wakogel LC-5K, spherical silica gel, was packed in a 33 cm × 2 mm I.D.

glass tube (Kyowa Seimitsu, Tokyo, Japan) by suspending the gel in water. Water was circulated through a jacket surrounding the column by a water-bath circulator (Model BT-35, Yamato Scientific) to maintain a temperature of 30°. A mobile phase, acetonitrile—3% aqueous ammonia (pH 10.0 by HCl) (85:15, v/v), was pumped through the column at a rate of 0.31 ml/min by a high-speed pump (Mitsumi Scientific Model SF-0396-57). Samples were applied via the injection port at the top of the column while the flow was stopped. The effluent from the column was monitored by a spectrophotometric detector (Shimadzu Model SPD-1, or Hitachi Model 634-0513) for absorbance at 277 nm, recorded with an Ohkura desk-top recorder.

RESULTS

The mobile phase for HPLC was chosen on the basis of a preliminary experiment for the separation of catecholamine-O-sulfates (3- and 4-O-monosulfates of DM, norepinephrine and epinephrine) on a silica gel thin layer [9]. The resolution of the peaks varied with the injection volume, and a volume of 5 μl was found to be sufficient for general use (Fig. 1). The peak height in absorbance was proportional to the amount of the conjugate from 50 ng (Fig. 2) to 5 μg.

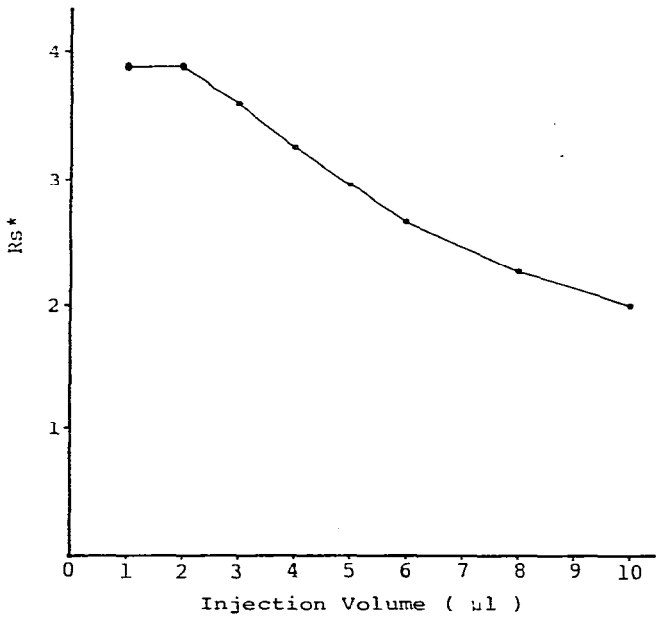

Fig. 1. Effect of injection volume on the resolution of dopamine-O-sulfates in HPLC. *Resolution value (Rs) was represented by the equation:

$$Rs = \frac{\text{interval between two dopamine-O-sulfate peaks}}{\text{mean band width of two dopamine-O-sulfate peaks}}$$

Conditions: column, Wakogel LC-5K (33 cm × 2 mm I.D.); eluent, acetonitrile—3% aqueous ammonia (pH 10.0 by HCl) (85:15, v/v); flow-rate, 0.31 ml/min; temperature, 30°.

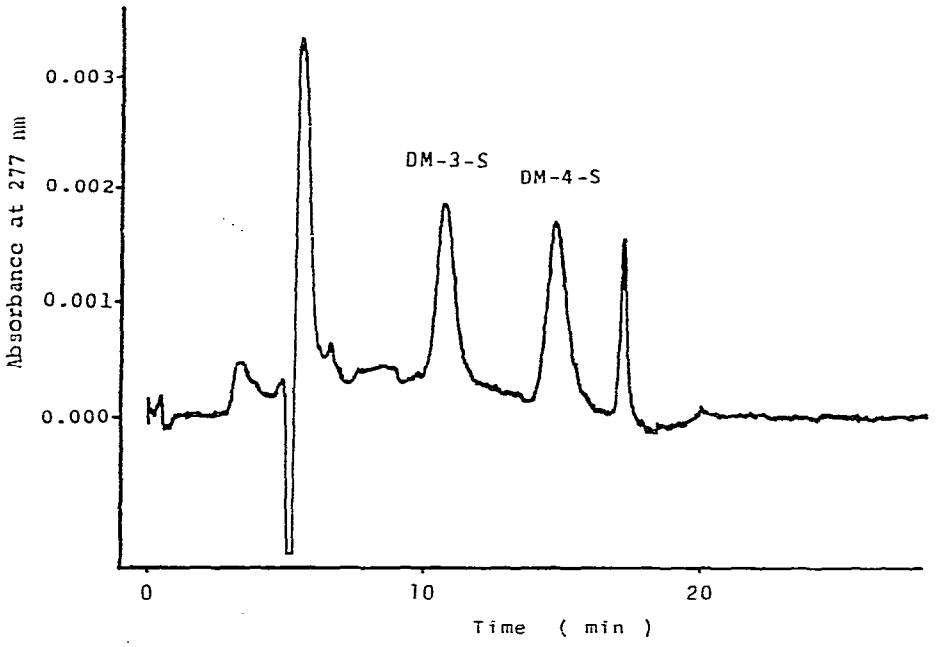

Fig. 2. High-performance liquid chromatogram of standard dopamine-O-sulfates. Five μl containing 50 ng each of dopamine-3-O-sulfate (DM-3-S) and dopamine-4-O-sulfate (DM-4-S) were injected and the eluate from the column was monitored by a Shimadzu spectrophotometric detector Model SPD-1. Other conditions are the same as in Fig. 1. The peaks other than those of DM-3-S and DM-4-S observed in the chromatogram were caused by the water used as the solvent for the standard dopamine-O-sulfate solutions.

Other constituents of urine, together with metabolites of L-DOPA, DM and dihydroxyphenylacetic acid (DOPAC), interfered when the sample urine was analysed directly. A Dowex 50W (H^+) column and a Dowex 1 (Cl^-) column removed these constituents through the sample preparation procedure. In order to protect the sulfates from hydrolysis on the column of Dowex 50W, a strong cation-exchange resin, chilled water was used for the elution.

Recoveries through the whole procedure of DM-3-S (25 or 50 μg) and DM-4-S (5 or 10 μg) added to 1 ml of urine were 96 ± 2.9% (S.D.) and 93 ± 3.0% (S.D.), respectively; these values were used for correction purposes.

The chromatogram obtained from the 2—4 h urine of a subject after the intake of 0.5 g of L-DOPA is depicted in Fig. 3. The accuracy of the amount of DM-3-S or DM-4-S estimated by HPLC was confirmed by the coincidence of the amounts in the DM-O-sulfate fractions on HPLC determined by two methods: (i) rechromatography of sulfate fractions, and (ii) fluorometric determination [10, 11] of liberated DM after acid hydrolysis of the O-sulfates (Fig. 4).

DM-3-S was detected in the 0—2 h, 2—4 h and 4—6 h urine; the largest amount was observed in 2—4 h urine in all cases, as shown in Fig. 5. Almost the same patterns were obtained for DM-4-S in a smaller amount (Fig. 6).

During 6 h, 6.6 ± 1.3% (S.D.) of orally administered L-DOPA (0.5 g) was excreted as DM-3-S in urine of normal subjects tested, while 0.9 ± 0.2% (S.D.) was excreted as DM-4-S. The ratio of DM-4-S to total DM-O-sulfates excreted in urine was 12.4 ± 0.56% (S.D.) for 0—2 h urine, 11.4 ± 0.69% (S.D.) for 2—4 h urine and 11.4 ± 0.63% (S.D.) for 4—6 h urine (the mean value during 6 h was 11.7 ± 0.58%).

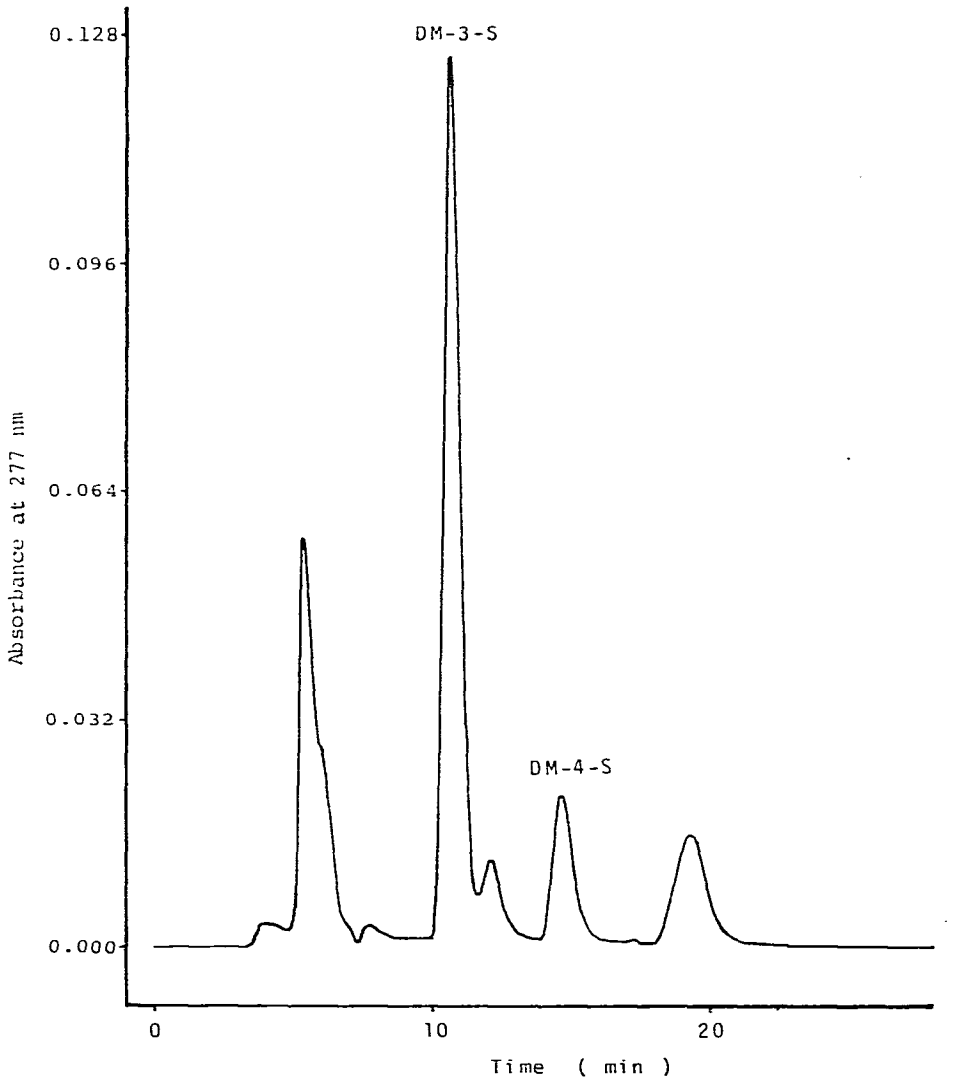

Fig. 3. High-performance liquid chromatogram of a urine sample. Injection: 5 μl of the sample preparation from the 2—4-h urine of a subject after administration of 0.5 g of L-DOPA. The conditions for HPLC were the same as in Fig. 2.

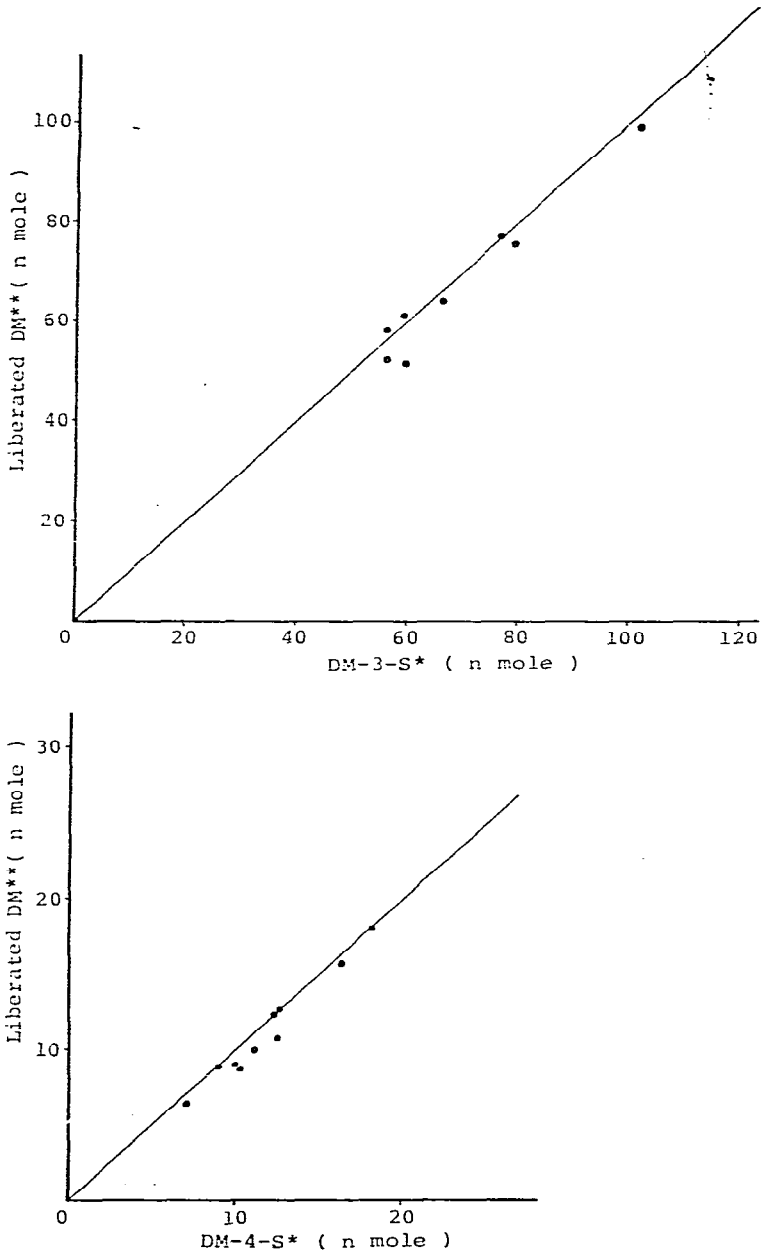

Fig. 4. Correlation between the amounts in the dopamine-O-sulfate fractions in HPLC determined by two methods. The fractions corresponding to each DM-O-sulfate in HPLC were collected after injection of 5 μl of several urine samples chosen at random. The collection was duplicated if necessary. Each fraction was evaporated and dissolved in 100 μl of water. *The amount of DM-O-sulfate in this solution was determined by the present HPLC method. **An aliquot of the solution was added to 1 ml of 0.1 *N* hydrochloric acid containing ascorbic acid (0.1 mg/ml) and EDTA·2Na (0.05 mg/ml), heated at 100° for 30 min, and the liberated dopamine was determined fluorometrically [10, 11]. The results were corrected for the efficiency of the hydrolysis procedure obtained by using standard dopamine-O-sulfate. The lines drawn in the figures represent the theoretical correlation.

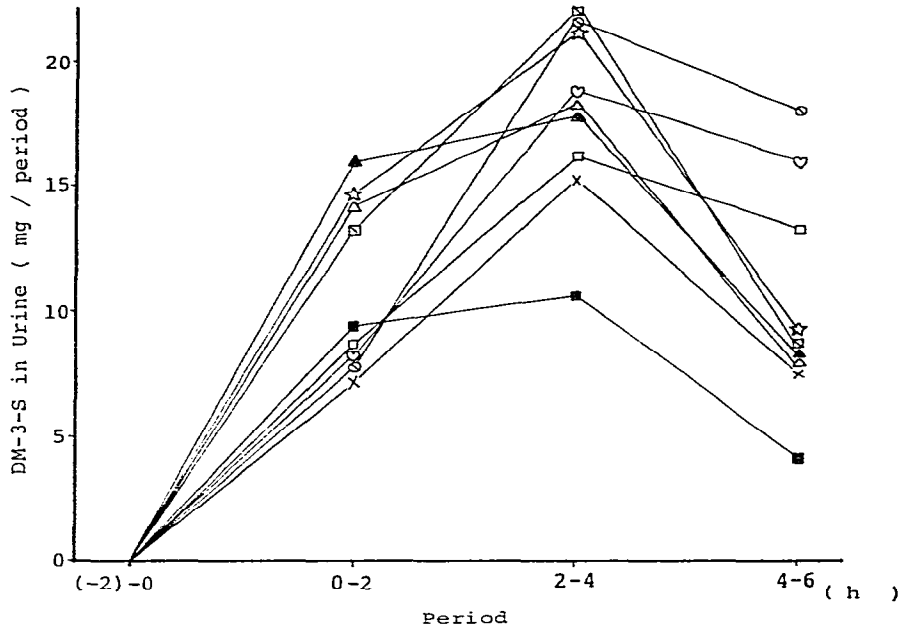

Fig. 5. Excretion of dopamine-3-O-sulfate after administration of 0.5 g of L-DOPA. The various symbols represent the individuals from whom samples were obtained.

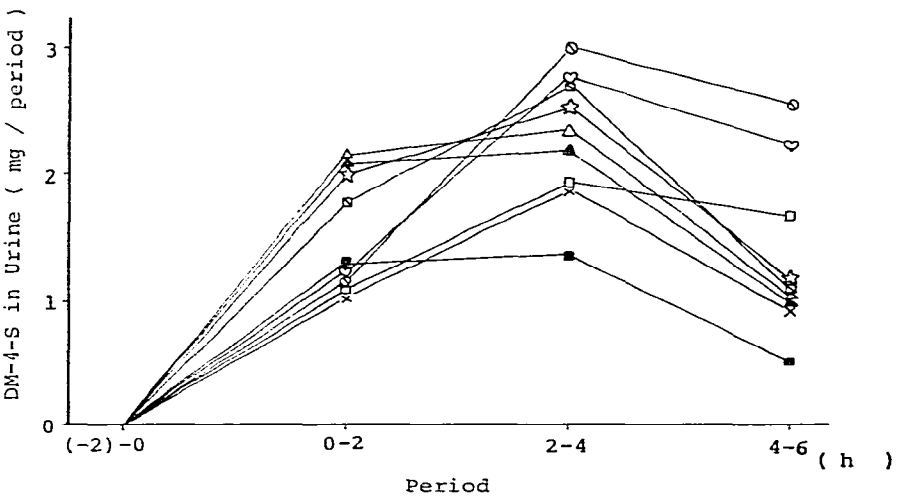

Fig. 6. Excretion of dopamine-4-O-sulfate after administration of 0.5 g of L-DOPA. Symbols are the same as in Fig. 5.

DISCUSSION

To determine urinary DM-O-sulfates in patients administered L-DOPA, Jenner and Rose [6] added ^{35}S-labeled DM-O-sulfates to 50 ml of urine sample for the correction of recoveries, concentrated the sample by lyophilisation, and separated the sulfates by 60-h elution on a Dowex 1 (CH_3COO^-) column (35 cm × 1.4 cm I.D.). They lyophilised 100 ml of each fraction of the sulfates, hydrolysed with acid and measured liberated DM by the trihydroxyindole

method. The method of Bronaugh et al. [8] is also tedious and time-consuming, as described in our introduction.

In the present work, high resolution and sensitive detection of the two DM-O-sulfates was achieved by silica-gel HPLC. Impurities of the peaks from urine were proved to be easily removed by sample clean-up on small columns. Consequently, only 1 ml of urine and 3.5 h were required for the whole procedure. The columns for sample preparation were easily made and regenerated, and when ten pairs of these columns were simultaneously used, as many as twenty samples were analysed per day.

So far as it has been investigated, the present method is simple, rapid, sensitive, accurate and reproducible and therefore suitable for the routine assay of DM-3-S and DM-4-S in urine of subjects administered L-DOPA.

In the present work the time course of urinary excretion of the conjugates after L-DOPA administration was first investigated, and DM-3-S was found to be the major sulfate of DM even in normal subjects. The higher ratio of DM-4-S to total DM-O-sulfates excreted in 0—2 h urine than in 2—4 h urine found for all subjects might be caused by the difference in DM-O-sulfate-producing organs [7]. The ratio of DM-4-S to total DM-O-sulfates during the 6 h following administration of L-DOPA was 11.7 ± 0.58% (S.D.), demonstrating the small range of variation in normal subjects. The ratios of 24-h urine of Parkinsonian patients reported by other workers were quite different from each other (16.3% by Jenner and Rose [6] and 4.9% by Bronaugh et al. [8]). It would be of interest to study the time course of urinary excretion of these DM-O-sulfates from Parkinsonian patients using the present method.

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