[Chem. Pharm. Bull. 29(7)2086—2089(1981)]

Improved Synthesis of Sulfoconjugate Isomers of Norepinephrine and Epinephrine, and Separation of All Sulfoconjugates of Catecholamines by Thin-Layer and High-Performance Liquid Chromatography[1)]

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(Received December 3, 1980)

A method for the synthesis of 3-O- and 4-O-sulfoconjugates of norepinephrine and epinephrine was developed by the reduction of the corresponding sulfates of noradrenalone and adrenalone. Simultaneous separation of the six kinds of sulfoconjugates of catecholamines was achieved by thin-layer chromatography and high-performance liquid chromatography.

Keywords——catecholamine; dopamine; norepinephrine; epinephrine; sulfoconjugate; conjugate; sulfate; synthesis; thin-layer chromatography; high-performance liquid chromatography

Together with free forms of catecholamines (CA), CA conjugates have been found in urine, blood and tissues. From the results of acid hydrolysis, the main conjugates have been considered to be O-sulfates (CA-S).[2-5)] Jenner *et al.*[6)] synthesized dopamine-3-O-sulfate (DM-3-S) and dopamine-4-O-sulfate (DM-4-S) by a direct sulfation of dopamine (DM) and developed a method for their determination by separation on an ion-exchange resin column followed by fluorometric measurement. They found that the ratio of the isomers formed from free DM after incubation with the supernatant of the homogenate of brain and liver differed between the tissues. They also showed that both sulfoconjugates of DM were present in the urine of patients with Parkinson's disease after the administration of L-3,4-dihydroxyphenylalanine (L-DOPA).[7)]

The separative determination of the sulfoconjugate isomers of norepinephrine (NE) and epinephrine (E) has not yet been achieved. In order to study these sulfoconjugates,authentic CA-S samples and a good separation method were needed. Syntheses of the sulfoconjugates of NE and E were reported by Wang *et al.*[8)] and Imai *et al.*,[9)] respectively. However, definitive syntheses of the 3-O- and 4-O-sulfates from the known 4-O- and 3-O-monobenzylethers of 3,4-dihydroxyacetophenone were required, so the methods were rather complicated and laborious.

In this report we describe improved syntheses of sulfoconjugates of NE and E and the separation of CA-S by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

Synthesis of Sulfoconjugates of NE and E

In preliminary experiments, we found that the sulfoconjugates of NE and E were not obtained by sulfating NE and E directly. This was probably because these benzylalcohols were easily decomposed under the reaction conditions. Therefore, we developed methods involving the reduction of the corresponding sulfates of noradrenalone (IIa) and adrenalone (IIb) (Chart 1).

IIa hydrochloride and IIb hydrochloride were prepared from 4-chloroacetylcatechol (I) by reaction with ammonia and methylamine, respectively, according to the method of Stolz.[10)]

Noradrenalone-3-O-sulfate (IIIa) and noradrenalone-4-O-sulfate (IVa) were synthesized by treating IIa hydrochloride with chlorosulfonic acid at below 0°C and purifying the product

on a Dowex 50W column. Adrenalone-3-O-sulfate (IIIb) and adrenalone-4-O-sulfate (IVb) were synthesized in similar ways.

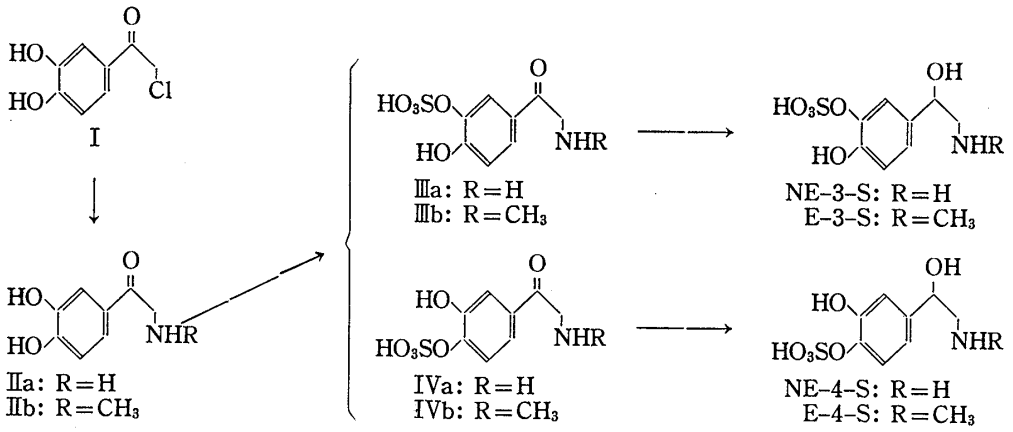

Chart 1. Synthetic Route for the Sulfoconjugates of NE and E

Reduction of these keto-sulfates, IIIa, IVa, IIIb, and IVb, with $NaBH_4$ gave *dl*-norepinephrine-3-O-sulfate(NE-3-S), *dl*-norepinephrine-4-O-sulfate(NE-4-S), *dl*-epinephrine-3-O-sulfate (E-3-S) and *dl*-epinephrine-4-O-sulfate (E-4-S), respectively.

The sulfates of NE and E were identified by TLC comparison with the authentic samples which had been prepared previously.[8,9] *Rf* values on TLC with the upper layer of butanol–acetic acid–water (4: 1: 5 by volume, solvent A) for the compounds obtained in each step are listed in Table I.

Table I. *Rf* Values on Silica Gel TLC for the Compounds obtained in Each Step of the Syntheses of CA–S

Compound	*Rf*[a]	Compound	*Rf*[a]
I	0.88	DM-3-S	0.32
IIa	0.42	DM-4-S	0.24
IIb	0.38	NE-3-S	0.24
IIIa	0.31	NE-4-S	0.18
IVa	0.25	E-3-S	0.21
IIIb	0.26	E-4-S	0.16
IVb	0.22		

a) Samples were developed on silica gel TLC plates (Kieselgel G-60, Merck) with solvent A (see the text). The compounds except for I were detected by spraying 0.2% (w/v) ninhydrin in ethanol–2 M acetic acid (19: 1, v/v) and heating the plate with hot air for about 2 min. I was detected by exposure of the plate to I_2 vapor.

Separation of CA-S by TLC

The 3-O- and 4-O-sulfate isomers of each CA were separated with solvent A, but simultaneous separation of all CA-S was not achieved because of their similar *Rf* values (Table I). However, a mixture of butanol, isopropanol and 1.7M aqueous ammonium buffer (adjusted to pH 10.0 with HCl) (1: 5: 5 by volume, solvent B) gave quite different *Rf* values and good resolution (Fig. 1).

Separation of CA-S by HPLC

In the light of the results of TLC, the HPLC system was based on a silica gel column and weakly alkaline eluent with detection by a UV monitor. After preliminary investigations, good separations were obtained (Fig. 2) under the following conditions: column, LiChrospher SI 100 (5 μm, 2 mm i.d. × 53 cm glass tube); eluent, acetonitrile–1.5 M aqueous ammonia

(adjusted to pH 10.0 with HCl) (85: 15, v/v); flow rate, 0.11 ml/min; temperature, 30°C; spectrophotometric detector (model SPD-1, Shimadzu 277 nm).

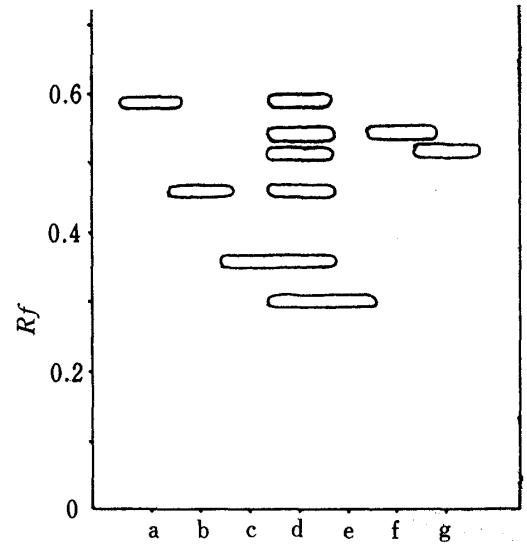

Fig. 1. Thin-Layer Chromatogram of CA-S

Samples were developed on a silica gel TLC plate with solvent B (see the text). The plate was heated at 120°C for 10 min to remove ammonia. CA-S were detected by spraying the plate with ninhydrin reagent and heating. Samples: a, DM-3-S; b, DM-4-S; c, E-3-S; d, mixture of 6 kinds of CA-S; e, E-4-S; f, NE-3-S; g, NE-4-S.

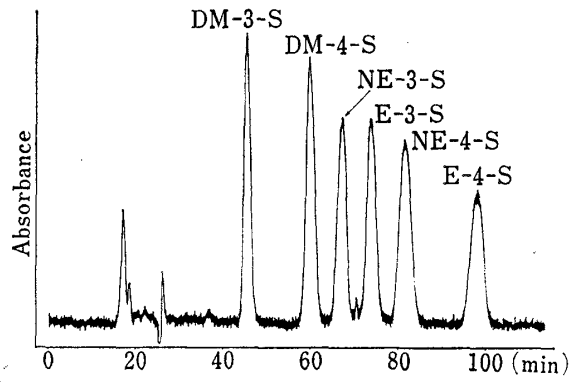

Fig. 2. Chromatogram of Standard CA-S

Details are given in the text. The peaks correspond to 150 ng of each sulfate.

Discussion

Although the specific biochemical sulfation of DM has been reported by some investigators,[6,7,11] such work on NE and E has not been reported, probably because of difficulty in synthesizing their 3-O- and 4-O-sulfates to provide authentic samples. The syntheses described in this paper are considerably simpler than those of Wang[8] and Imai.[9] In particular, 3-O-sulfates of NE and E are easily obtained in high yields without chromatographic separation.

By using the TLC method and HPLC method developed here, simultaneous separation of all sulfoconjugates of CA can be achieved, which should be valuable for metabolic studies on CA. We are now developing a sensitive analytical method for endogenous CA-S, which will be presented elsewhere.

Experimental[12]

Noradrenalone-3-O-sulfate (IIIa) and Noradrenalone-4-O-sulfate (IVa)——IIa hydrochloride (1.2 g) was added in small portions with stirring to 5 ml of chlorosulfonic acid in a 100 ml round-bottomed flask chilled below −45°C. The temperature was raised gradually with care to avoid the vigorous generation of hydrogen chloride gas, and the solution was kept at around −2°C for 1 h. The mixture was then chilled again and poured dropwise onto 25 g of cracked ice with vigorous stirring. The solution was stored at −20°C overnight. The crystals (IIIa) that precipitated were filtered off and recrystallized from water, 1.0 g, decomp. 250°C. *Anal.* Calcd. for $C_8H_9NO_6S$: C, 38.86; H, 3.67; N, 5.67. Found: C, 38.95; H, 3.62; N, 5.79.

The mother liquor was placed on a column (4.6 cm i.d. × 36 cm) of Dowex 50W (×8, 200—400 mesh, H^+ form) in a cold room at 6°C and eluted with water. The fractions were monitored by silica gel TLC with solvent A and the ninhydrin reagent. The fractions containing IVa (850—1000 ml), eluted after IIIa (650—750 ml), were collected and lyophilized (22 mg). This product was used for the synthesis of NE-4-S. A small portion of the product was recrystallized from water, decomp. 260°C. *Anal.* Calcd. for $C_8H_9NO_6S$: C, 38.86; H, 3.67; N, 5.67. Found: C, 38.85; H, 3.70; N, 5.80.

Adrenalone-3-O-sulfate (IIIb) and Adrenalone-4-O-sulfate (IVb)——From 2.5 g of IIb hydrochloride, 1.1 g of IIIb and 70 mg of IVb were obtained by a procedure similar to that described for the synthesis of

IIIa and IVa. IIIb: mp 247—247.5°C (dec.). IVb: mp 197—200°C (recrystallized from water). *Anal.* Calcd. for $C_9H_{11}NO_6S$ (IIIb and IVb): C, 41.38; H, 4.24; N, 5.36. Found for IIIb: C, 41.39; H, 4.15; N, 5.31. Found for IVb: C, 41.30; H, 4.17; N, 5.65.

***dl*-Norepinephrine-4-O-sulfate (NE-4-S)**——A suspension of 32 mg of IVa in 24 ml of dehydrated methanol–isopropanol (3: 5, v/v) was treated with 20 mg of $NaBH_4$ in several portions over 4 h with occasional stirring and then left for a further 3 h. The solution was evaporated to dryness. The residue was dissolved in 2 ml of water and 2 N HCl was added until the pH became about 2 (pH test paper). This solution was applied to a Dowex 50W column (H^+ form, 1.6 cm i.d. × 30 cm), which was eluted with water. The ninhydrin-positive fractions eluted after the acidic fractions were collected and concentrated *in vacuo*. The white crystals that precipitated were filtered off and washed with a small portion of ethanol, 22 mg, decomp. above 152°C. *Anal.* Calcd. for $C_8H_{11}NO_6S$: C, 38.55; H, 4.45; N, 5.62. Found: C, 38.66; H, 4.47; N, 5.74.

***dl*-Norepinephrine-3-O-sulfate(NE-3-S), *dl*-Epinephrine-4-O-sulfate(E-4-S) and *dl*-Epinephrine-3-O-sulfate (E-3-S)**——These compounds were synthesized by a procedure similar to that described for NE-4-S.

From 360 mg of IIIa, 220 mg of NE-3-S was obtained after recrystallization from water, decomp. 150°C. *Anal.* Calcd. for $C_8H_{11}NO_6S$: C, 38.55; H, 4.45; N, 5.62. Found: C, 38.74; H, 4.37; N, 5.76.

From 57 mg of IVb, 40 mg of E-4-S was obtained, mp 160—161°C (methanol). *Anal.* Calcd. for $C_9H_{13}NO_6S$: C, 41.06; H, 4.98; N, 5.32. Found: C, 41.08; H, 4.97; N, 5.32.

From 400 mg of IIIb, 220 mg of E-3-S was obtained after recrystallization from water–ethanol (1: 2, v/v), mp 158—160°C (dec.). *Anal.* Calcd for $C_9H_{13}NO_6S$: C, 41.06; H, 4.98; N, 5.32. Found: C, 41.16; H, 5.03; N, 5.63.

References and Notes

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12) All the melting points are uncorrected.

[Chem. Pharm. Bull. 29(7)2089—2092(1981)]

Studies on the Hydroxylation of Phenylalanine by the Ascorbic Acid–Hydrogen Peroxide System

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(Received January 26, 1981)

When phenylalanine was treated with the ascorbic acid–hydrogen peroxide system in acetate buffer (pH 4.0), *o*-tyrosine, *m*-tyrosine and *p*-tyrosine were identified as hydroxylated products.

The hydroxylation of phenylalanine in the ascorbic acid–hydrogen peroxide system was prevented by radical scavengers, *e.g.*, potassium iodide, potassium bromide, sodium thiocyanate, sodium formate, mannose, ethyl alcohol, superoxide dismutase and Tiron.

The possibility is discussed that the active species responsible for the hydroxylation of phenylalanine in the ascorbic acid–hydrogen peroxide system is the hydroxyl radical.