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# Gas Chromatographic Determination of 3,4-Dihydroxyphenylalanine (DOPA) in Urine

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Urinary 3,4-dihydroxyphenylalanine (DOPA) was extracted with alumina after removal of the fatty substances by shaking with ether–benzene (5:2), converted to *n*-butyl ester with *n*-butanol–HCl (5N) at 100° for 20 min, trifluoroacetylated with trifluoroacetic anhydride in ethyl acetate and determined by gas chromatography using electron capture detector. The overall recoveries of the added DOPA (50, 75, 100, and 150 ng) from urine (2 ml) were 66.0% ±3.55% ($n=8$).

The pressence of DOPA in normal human urine was confirmed by gas chromatography-mass spectroscopy.

L-3,4-Dihydroxyphenylalanine (L-DOPA), an immediate precursor for catecholamines, has been determined colorimetrically (with amino acid analyzer[2]) and fluorometrically (THI,[3] ED[4] or formaldehyde condensation[5] methods), however, none of those are sensitive or selective enough for determining the minute amount of DOPA present in natural biological fluids. In the studies on L-DOPA metabolism, the authors had used a gas chromatographic method in which the amino acid was converted to a trifluoroacetyl (TFA) oxazolone[6] and determined with an electron capture detector (ECD),[7] even when the sensitivity was about one fifth to that of epinephrine.

In this report we investigated some esters of DOPA to obtain a more sensitive and selective TFA derivative for gas chromatographic determination with ECD, together with isolation of DOPA from urinary samples.

## Experimental

**Apparatus and Condition**——A Shimadzu Model GC-3A or GC-4AP gas chromatograph equipped with ECD was used. The glass tube (3 m or 2.5 m×4 mm *i.d.*) was packed with 1% or 1.5% GE-XF 1105 on Chromosorb W (60—80 mesh). For GC-3A type: injection port temperature, column temperature and detector temperature were 166°; nitrogen flow rate, 60 ml/min. For GC-4A type; injection port temperature, 195°; column temperature, 190°: detector temperature, 195°; nitrogen flow rate, 52 ml/min. A Shimadzu LKB-9000 gas chromatograph-mass spectroscope was used. Mass spectra were measured under following conditions: electron energy, 70 eV; ionization source temperature, 250°; separator temperature, 200°.

**Materials**——L-DOPA (Daiichi Seiyaku Co., Ltd.), dopamine hydrochloride, *dl*-norepinephrine (Tokyo Kasei Co., Ltd.), *l*-epinephrine (Merck Co., Ltd.), isodrin (Sankyo Co., Ltd.) and dieldrin (Gaschro Cogyo Co., Ltd.) were used.

**Preparation of TFA Derivatives of DOPA Esters**——The 0.2N acetic acid solution containing some hundred ng of DOPA was evaporated to dryness at 40—50°. The residue was dried over potassium hydroxide

1) Location: *Hongo 7-3-1, Bunkyo-ku, Tokyo.*
2) T.A. Hare and W.H. Vogel, *Biochem. Med.*, 4, 277 (1970).
3) H.E. Spiegel and A.E. Tonchen, *Clin. Chem.*, 16, 763 (1970).
4) F. Eichhorn, A. Rutenberg, and E. Kott, *Clin. Chem.*, 17, 296 (1971).
5) N. Seiler and M. Wiechmann, *Z. Physiol. Chem.*, 337, 229 (1964).
6) The structure of the compound will be published in a near future.
7) K. Imai, M. Sugiura, H. Kubo, Z. Tamura, K. Ohoya, N. Tsunakawa, K. Hirayama, and H. Narabayashi, *Chem. Pharm. Bull.* (Tokyo), 20, 759 (1972).

under a reduced pressure for 10 min and dissolved in 0.5 ml of anhydrous alcohol (methyl, ethyl, *n*-propyl or *n*-butyl alcohol) saturated with HCl gas and heated at 60° or 100° for several minutes. The reaction mixture was evaporated under a reduced pressure at 60° (57—64°). The residue was dried over potassium hydroxide under a reduced pressure for 5 min, dissolved in 100 $\mu$l of ethyl acetate and 10 $\mu$l of trifluoroacetic anhydride and reacted at room temperature for 5 min.

**Gas Chromatography of DOPA and Catecholamine Derivatives**——The above reaction mixture was diluted with *n*-hexane containing an internal standard (0.25 ng dieldrin/$\mu$l of *n*-hexane) and one $\mu$l of the solution was immediately injected to a gas chromatograph. In the case of catecholamines the treatment was the same as above but omitting the esterification.

**Procedure for the Isolation of DOPA from Solutions**——To 15 ml of 0.25M triethylamine carbonate buffer (pH 6) solution of DOPA was added 1 ml of 0.2M EDTA and the pH was adjusted to about 6.5 with 4N ammonia. Some hundred mg (100, 200, 400, 800 or 1000 mg) of acid washed alumina was added to the solution and the pH was adjusted to 6.5, 7, 8, 8.5 or 9 with 4N ammonia. The solution was stirred mechanically with a glass rod for 10 min. The alumina was taken to a glass chromatographic tube with the aid of water. The adsorbed DOPA was eluted with various concentration of acetic acid in methanol or in water.

**Isolation and Gas Chromatagraphy-Mass Spectroscopy of Human Urinary DOPA**——Eight hundred ml of a normal human urine was divided to 8 portions. Each aliquot was diluted to 300 ml with distilled water and treated with 1 g of alumina according to the standard procedure. The eight eluates from alumina were combined and evaporated to dryness, dissolved in 5 ml of 0.4N acetic acid and extracted with 5 ml of ether–benzene (5: 2) 3 times. The water phase was evaporated to dryness and converted to *n*-butyl ester-TFA derivative as described in the standard procedure. Three $\mu$l of the reaction mixture was injected to a gas chromatograph-mass spectroscope.

## Result and Discussion

### Preliminary Investigation

As show in Table I, TFA derivative of DOPA *n*-butyl ester was clearly separated from TFA derivatives of catecholamines on a 1.5% GE-XF 1105 column, while methyl, ethyl and *n*-propyl derivatives were not sufficiently separated. Since DOPA and catecholamines were isolated simultaneously with alumina, the TFA derivatives of L-DOPA *n*-butyl ester was used thereafter. Dieldrin was found to be a better internal standard than isordin[6] in this case.

Table I. Relative Retention Times of TFA Derivatives of DOPA Esters and Related Compounds

| Compounds | Relative retension times | Compounds | Relative retension times |
|---|---|---|---|
| DOPA | 0.148 | Norepinephrine | 0.398 |
| Epinephrine | 0.213 | DOPA *n*-propyl ester | 0.444 |
| Dopamine | 0.292 | Isodrin | 0.556 |
| DOPAmethyl ester | 0.296 | DOPA *n*-butyl ester | 0.583 |
| DOPA ethyl ester | 0.343 | Dieldrin | 1.00 (10.8 min) |

gas chromatographic condition: 1.5% GE–XF 1105 (4 mm×2.5 m), 190°

DOPA was esterified incompletely at 60° for 30 min with *n*-butanol saturated with HCl gas (5N) as shown in Fig. 1 while esterification at 100° was accomplished for 20 min. Roach and Gehrke also reported that all the protein amino acids except isoleucine were esterified with *n*-butanol–HCl (3N) at 100° for 15 min.[8]

In this condition catecholamines were degraded and could not be determined. The degradation products did not disturb the determination of DOPA.

A linear relationship between the amount of DOPA and peak height ratio of the derivative of DOPA to dieldrin was obtained in the range of 25 ng to 100 ng as shown in Fig. 2 using the above condition.

8) D. Roach and C.W. Gehrk, *J. Chromatog.*, **44**, 269 (1969).

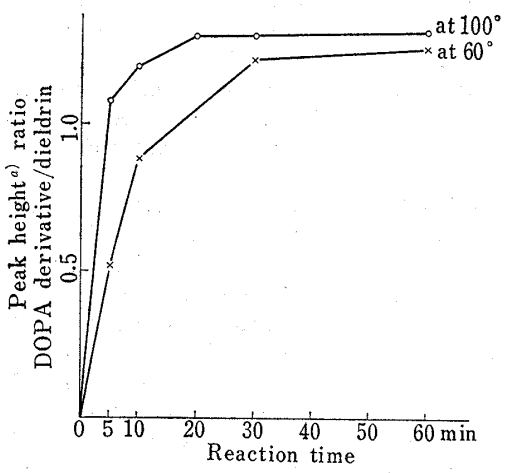


Fig. 1. Esterification of DOPA with *n*-BuOH–HCl

*a*) The peak height measured is the distance from the preceding foot to the top of peak.

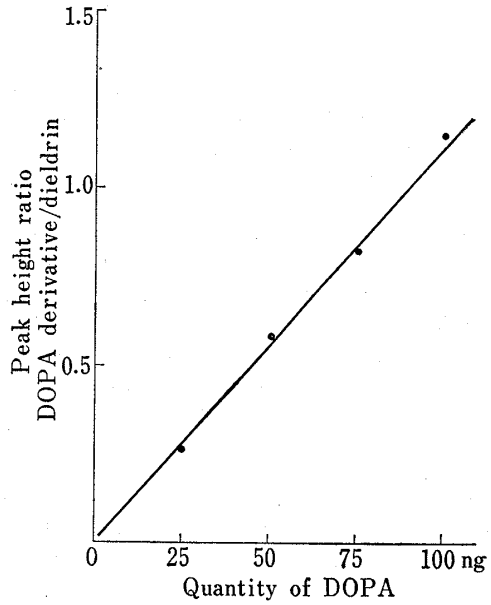


Fig. 2. Calibration Curve for TFA Derivative of DOPA *n*-Butyl Ester

When 250 ng of DOPA adsorbed on 0.1 g of alumina was eluted with 1 ml of 1N acetic acid in methanol[6] and 1 ml of 0.2N, 0.5 ml of 0.4N, 0.8N, and 1N acetic acid, the total recoveries of DOPA were 20, 41, 55, 50, and 53% respectively. And with the higher concentration of acetic acid, the amount of residual substances became large to reduce the heights of gas chromatographic peaks. So the elution solvent was decided to be 0.4N acetic acid.

By eluting with 0.4N acetic acid (5 ml/g of alumina), the total recovery did not change when the amount of alumina varied between 0.1 g and 1 g, but more than 0.5 g of alumina was required in the analysis of urinary DOPA.

An added DOPA was poorly recovered from some concentrated urine specimens since the urinary fatty substances which was adsorbed on alumina disturbed the final gas chromatographic analysis. The difficulty was overcomed by extracting them with ether–benzene (5: 2).

The optimum pH range at the time of adsorption on alumina was between 8 and 9 (total recovery 51—54%), and below pH 7.5 the recovery was low (pH 7.5, 46%; pH 7, 30%; and pH 6, 11%).

**Standard Procedure for the Determination of DOPA in Human Urine**

Urine (2—5 ml) was acidified (pH 1) with hydrochloric acid and defatted by shaking two times with equal volume of ether–benzene (5: 2). To the water phase was added 1 ml of 0.2M EDTA and the pH was adjusted to about 6 with 4N ammonia. One g of acid washed alumina was added to the solution and the pH was adjusted to 8.5 with 4N ammonia. The solution was stirred mechanically with a glass rod for 10 min. Then the alumina was taken into a glass tube (1×10 cm) with the aid of distilled water and washed with 1 ml of 0.4N acetic acid. The adsorbed DOPA was eluted with the successive 3 ml of 0.4N acetic acid.

Table II. Recovery of L-DOPA added to Normal Human Urine (2 ml)

| L-DOPA added (ng) | 50 | 75 | 100 | 150 |
|---|---|---|---|---|
| Recovery (%) | 71.0 71.3 | 62.1 63.5 | 65.6 62.6 | 67.1 65.1 |

$\bar{x}$=66.0 $\sigma$=3.55

The eluate combined with 2 ml of methanol was evaporated to dryness under a reduced pressure and dried over sodium hydroxide for 5 min. The residue was dissolved in 1 ml of *n*-butanol saturated with HCl gas (5N) and esterified at 100° for 20 min in a tightly stoppered vial. The reaction mixture was evaporated at 60° under a reduced pressure and dried over sodium hydroxide for 10 min. The residue was dissolved in 100 μl of ethyl acetate and 10 μl of trifluoroacetic anhydride and reacted at room temperature for 5 min. The reaction mixture was diluted with 0.5 ml of *n*-hexane containing dieldrin (0.25 ng/μl of *n*-hexane) and one μl of the solution was injected to a gas chromatograph equipped with an ECD. The content of DOPA was estimated from the calibration curve in Fig. 2.

The recoveries of the added DOPA from urine specimens are shown in Table II.

The chromatogram thus obtained from a normal human urine is shown in Fig. 3 demonstrating the presence of DOPA.

The occurrence of DOPA in normal human urine has been suggested but not clearly demonstrated[9–11] on account of its low excretion. This was further confirmed by mass spectroscopy combined with gas chromatography. The mass spectrum of the peak was the same as that of the authentic DOPA derivative as shown in Fig. 4 and 5.

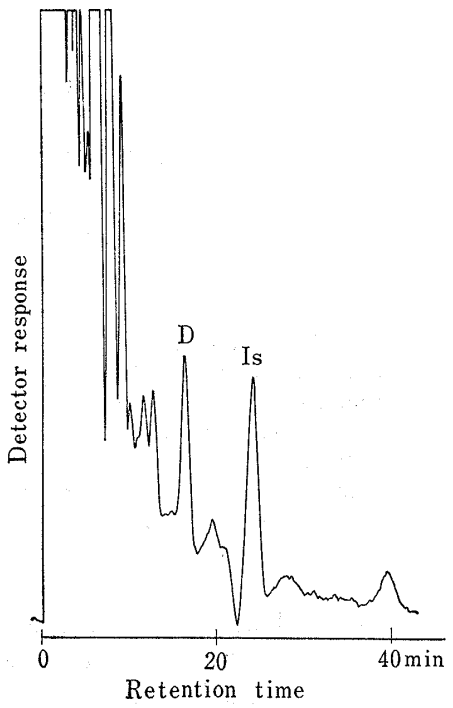


Fig. 3. Gas Chromatogram of a Normal Human Urine (2 ml)

condition: Shimadzu GC-3AE, 1% GE-XF 1105 on Chromosorb W(60—80 mesh) (4 mm × 3 m) 166°
nitrogen flow rate, 60 ml/min
Is: dieldrin (internal standard)
D: DOPA *n*-butyl ester-TFA deriv

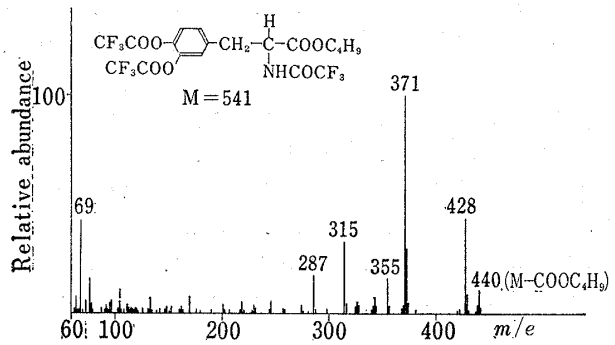


Fig. 4. GC-Mass Spectrum of TFA Derivative of Authentic DOPA *n*-Butyl Ester

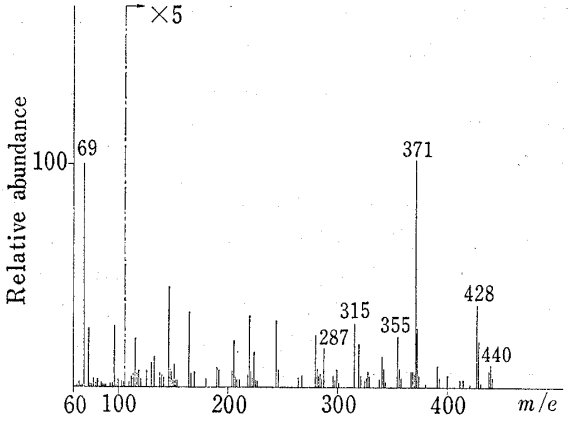


Fig. 5. GC-Mass Spectrum of TFA Derivative of Urinary DOPA *n*-Butyl Ester

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9) R. Imaizumi and K. Sano, *Folia Endocrinol. Jap.*, **33**, 932 (1958).
10) S. Kawai and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **16**, 1091 (1968).
11) D.D. Clark, S. Wilk, S.E. Gitlow, and M.J. Franklin, *J. Gas. Chromatog.*, **1967**, 307.