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Gas Chromatography of the Catecholamines as Their Trifluoroacetates in Urine and Tumor

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Gas chromatographic assay of the catecholamines in urines and tumor was successfully carried out using the procedure described in the previous paper.[5)] Gas chromatographic method was superior to fluorometry as regards selectivity. Each catecholamine was analysed simultaneously and the whole pattern of chromatogram permitted a rapid, simple diagnostic evaluation. Moreover the resolving power of gas chromatography led to a finding of a co-existing unknown substance, which had the identical retention time to dopa and existed in a significant amount in normal urine, as well as in the urine of a patient with pheochromocytoma.

Engel, *et al.*[2)] confirmed that hypertension developed due to pheochromocytoma was associated with norepinephrine. Mason, *et al.*[3)] found that the catecholamines were largely excreted in urine not only from the patient with pheochromocytoma, but also from the patient with neuroblastoma. These findings evaluated the clinical and diagnostic significance of the determination of the urinary catecholamines. A number of investigators have reported the fluorometric assay of urinary catecholamines, while there have been no reference about a gas chromatographic determination except only one recent report,[4)] in which Clark, *et al.* described the determination of dopamine in urine at a nanogram level. In the previous paper,[5)] we reported the microdetermination of the catecholamines by gas chromatography as their trifluoroacetates using an electron capture detector. In the present paper, the technique was applied with advantage to the catecholamines in urine and tumor tissue extract.

Experimental

Apparatus and Condition——Apparatus and gas chromatographic conditions employed here are essentially the same as described in the previous paper.[5)]

Isolation of Catecholamines from Urine——The following procedures were used in the isolation of the catecholamines from urines:

(1) Acidify 50 ml of a filtered urine to about pH 1 with 6 N HCl and boil for 20 min under reflux to hydrolyze the conjugated amines.

(2) After cooling, neutralize the solution to about pH 6.5 with 4 N NH_4OH under a magnetic stirring.

(3) Add 2 ml of 0.2 M EDTA-Na_2 and 2 g of acid treated Al_2O_3.

(4) Adjust pH of the mixture to about 8.4 with 1 N NH_4OH and shake gently for 5 min to adsorb the catecholamines onto Al_2O_3.

(5) Allow to settle the mixture for several minutes and discard the supernatant.

(6) Transfer the sediment into a chromatographic tube by the aid of distl. H_2O and wash with H_2O.

(7) Elute the catecholamines from Al_2O_3 with 10 ml of 0.2 N AcOH and measure the volume of the eluate.

(8) Centrifuge if needed and add a drop of 0.5% acetylacetone aq. soln. to 0.5 ml aliquot of the eluate.

(9) Treat the mixture as described in previous paper[5)] for gas chromatographic analysis.

1) Location: *Hongo-7-chome, Bunkyo-ku, Tokyo.*

2) A. Engel and U.S. von Euler, *Lancet*, **259**, 387 (1950).

3) G.A. Mason, J. Hart-Mercer, E.J. Millar, L.B. Starng, and N. Wynne, *Lancet*, **273**, 322 (1957).

4) D.D. Clark, S. Wilk, S.E. Gitlow, and M.J. Franklin, *J. Gas Chromatog.*, **1967**, 307.

5) S. Kawai and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **16**, 699 (1968).

Extraction of Catecholamines from Tumor Tissue——One g of the tumor tissue was homogenized in a potter galss homogenizer containing 5 ml of 10% trichloroacetic acid. The homogenate was centrifuged for 10 min at 4000 rpm at a room temperature. The supernatant was decanted into a beaker and the residue was reextracted with 5 ml of 5% trichloroacetic acid. The combined extract was treated as described for urine from step (2).

Fluorometry——For measurement of epinephrine and norepinephrine, the trihydroxy indole reaction was carried out essentially according to the method of von Euler and Floding.[6] Measurement of dopamine was carried out essentially according to the procedure as described in "Fluorescence Assay[1] by Udenfriend.[7]

Results and Discussion

Clark, *et al.* made the first contribution[4] to gas chromatographic determination of urinary catecholamines. However, some fundamental differences exist between their and our reports in the following respects. Instead of QF–1, we chose GE–XF 1105 as a stationary phase which improved the separation of the catecholamines and was enough to distinguish epinephrine and dopa. They used an absolute calibration method on the determination, while we used an internal standard method with isodrin, the values obtained by which were more reliable. In their procedures,[4] alumina was added to an acidified urine sample into which the alumina dissolved considerably, and mixed vigorously with a magnetic stirrer which broke the alumina into fine powders. Such procedure prolonged the time for eluting of the amines and increased an intermixing quantity of the fine alumina powders into the eluate. To avoid these disadvantages, we added alumina after the neutralization of the urine sample to about pH 6.5 and the mixture was shaken gently by hand. Although attentions above were payed, it was difficult to prevent the intermixing of traces of alumina and/or aluminum ion into the eluate from the alumina column, which has been observed to decompose the catecholamines on the evaporation of the eluate to dryness. To circumvent this interference, a drop of 0.5% acetyl acetone aqueous solution was added. A working curve at a range from 12.5 to 50 μg of epinephrine was found to be lineary related to the quantity added in urine samples (Fig. 1). A 50 μg of epinephrine was added to each 50 ml of urine sample (about pH 1) and the recoveries were determined by both of gas chromatographic and fluorometric methods through the procedure described in the experimental section. The value obtained, as shown in Table I, was superior to that obtained by the batch adsorption procedure of Clark, *et al.*

Table I. Recovery[a] (%) of Epinephrine by the Procedure

	Gas chromatography	Fluorometry
1	81.4	75.4
2	80.6	76.9
3	73.5	76.9
4	86.8	79.6
5	82.1	75.5
6	82.4	75.5
7	75.4	71.9
8	82.5	71.9
9	78.6	70.3
10	78.9	75.5
Av.	80.2	74.9
St. Dev. (%)	3.8	2.8

a) Recovery tests were repeated by carrying ten identical urine samples containing 50 μg of added epinephrine

6) U.S. von Euler and I. Floding, *Acta Physiol. Scandinav.*, **33**, Suppl. **118**, 45 (1955).
7) S. Udenfriend, Fluorescence Assay in Biology and Medicine, **1962**, 137.

This technique was applied to the determination of the catecholamines in the urines of two patients (A and B) with pheochromocytoma, a patient (C) with hypertension and a normal person (D), and the tumor (T) removed from patient B. Fig. 2 shows the chromatogram obtained from the urine of patient B and that of normal subject D is illustrated in Fig. 3.

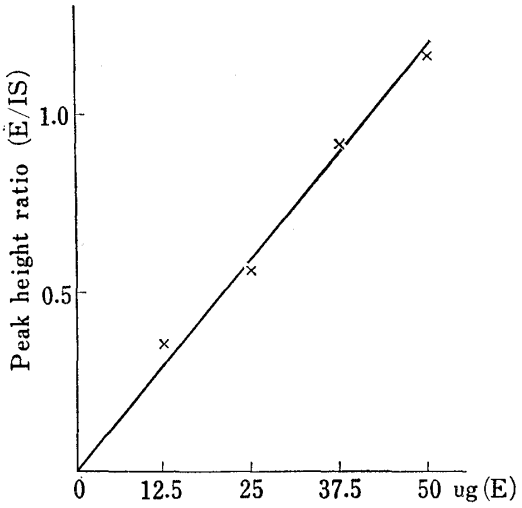

Fig. 1. Working Curve of Epinephrine (E) added in Urine Samples through the Whole Procedure

Isodrin was used as an internal standard (IS)

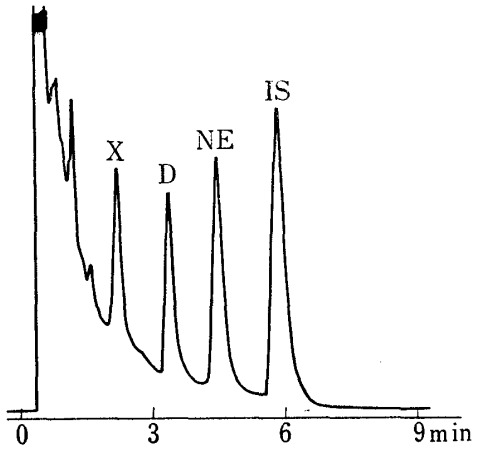

Fig. 2. Gas Chromatogram of Catecholamines in the Urine of Patient B with Pheochromocytoma

X: unknown, D: dopamine, NE: norepinephrine
IS: internal standard (isodrin)
Condition: 2% GE-XF 1105 on 80—100 mesh Gas-Chrom P glass column, 2.0 m×4 mm, 175° and about 80 ml of nitrogen per min.
Detecor: an electron capture detector

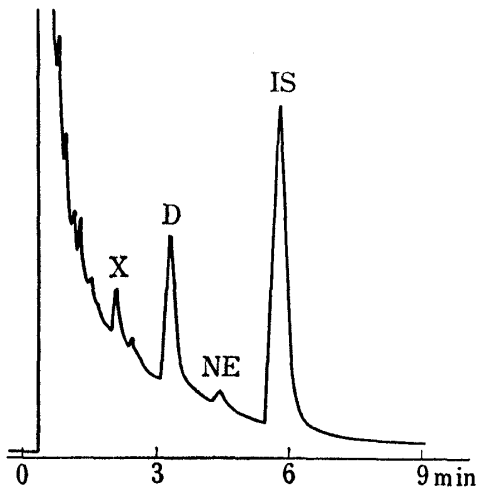

Fig. 3. Gas Chromatogram of Catecholamines in the Urine of Normal Person

X: unknown, E: epinephrine, D: dopamine, NE: norepinephrine, IS: internal standard (isodrin)
Condition and Detector: the same as Fig. 2

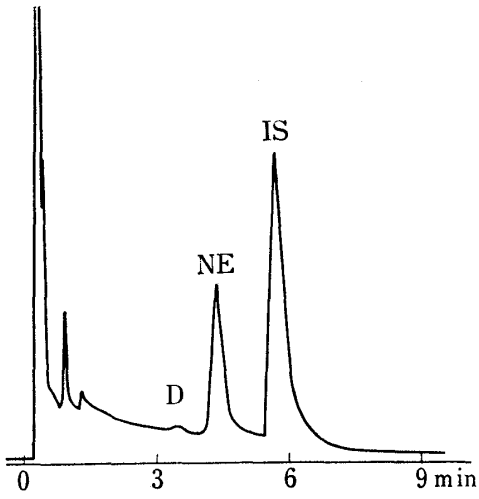

Fig. 4. Gas Chromatogram of Catecholamines in the Tumor removed from Patient B

D: dopamin, NE: norepinephrine, IS: internal standard (isodrin)
Condition and Detector: the same as Fig. 2

Gas chromatographic and fluorometric data are compared in Table II.

If one considers these data so far, it is clear that the urinary output of norepinephrine is elevated to abnormal amounts in pheochromocytoma. Dopamine is a normal constituent of human urine and excreted in high amounts in both of Fig. 2 and Fig. 3. In the urine of

Table II. Determination of Urinary Catecholamines (μg/day)

Smaples	Fluorometry			Gas chromatography		
	Norepine-phrine	Epine-phrine	Dopamine	Norepine-phrine	Epine-phrine	Dopamine
A	1342	—	452	1345	8	445
				1520	6.4	346
				1340	7.2	371
B1	1340	—	940	560	—	210
B2	1940	—	967	1620	—	540
				1550	—	563
B3	1910	—	983	1110	—	418
				1020	—	398
C	12.6	1.8	529	?	?	359
D	26.6	3.1	645	27.7	13.9	280
T (μg/g)	1710	—	—	1645	—	12

A and B: patients with pheochromocytoma
B1, B2 and B3: the urinary catecholamines occurring at different days in the patient B
C: a patient with hypertension
D: normal subject
T: a tumor in patient B

patient C with hypertension, normal norepinephrine output is observed, indicating that the patient C has no pheochromocytoma.

At the present time, gas chromatographic method is not enough to permit the exact determination of epinephrine in normal levels. The values shown in the column of epinephrine in Table II are only approximate. In practice, an injectable volume onto gas chromatograph is limited to a few microliters because an excess of trifluoroacetic anhydride spoils an electron capture detector and disturbs the separation of micro-amount of the catecholamines. To overcome this limit, we have been investigating the procedure so that the total sample or most of it may be applied to the column connecting with an electron capture detector.

Gas chromatographic method has several advantages over fluorometry. Each peak indicates reliable amount of catecholamine itself without the interference of other co-existing components. Fluorometry often gives unreliable informations, since the amounts of epinephrine and norepinephrine are calculated by the use of simultaneous equations, in which dopa also interferes. An additional point of interest in gas chromatography is that the amount of each catecholamine can be estimated simultaneously on the same chromatogram. In most cases, the whole pattern of chromatogram itself may have a clinical significance without precise quantitative estimation of each catecholamine. As shown in Fig. 2 and Fig. 3, the increase in the ratio of norepinephrine to dopamine in peak height, permits a rapid, simple diagnostic evaluation. Fig. 4 shows the separation of the amines in the tumor and the pattern reveals norepinephrine to be the only predominant catecholamine, which suggest that this tumor produces only norepinephrine because of an inability to methylate it to epinephrine. Moreover, coexisting unknown compounds, if any, may be discriminated on the same chromatogram. As observed in Fig. 2 or Fig. 3, the unknown peak X was found in a significant amount. In view of the correspondence of retention times so far, it is not unreasonable to assume the compound of peak X might be dopa itself. Dopa has not been found in urine except a few papers[4,8] and of great interesting is the presence of considerable amounts of dopa in the urines not only of a patient with pheochromocytoma, but also of a normal person.

8) W. von Studnitz, H. Kaser, and A. Sjoerdma, *New Engl. J. Med.*, **269**, 232 (1963).