[Chem. Pharm. Bull.
15(10)1493～1497(1967)]

UDC 615.361.452-07 : 543.544.25

190. Satoshi Kawai and Zenzo Tamura[*1] : Gas Chromatography of Catecholamines using Dimethyl Sulfoxide as an Effective Solvent for Trimethylsilylation.

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Separation of catecholamines by gas chromatographic technique requires the preparation of suitable derivatives. A procedure has been developed which involved the treatment of amine mixtures with hexamethyldisilazane in dimethyl sulfoxide followed by the addition of an aliphatic ketone. Hydroxyl groups are converted into trimethylsilyl ether and primary amines are converted into Schiff bases. Secondary amino group remains unchanged. The separations were carried out with a 7% DC 1107 column. Catecholamines extracted from bovine adrenal medulla were separated and estimated under these conditions and good reproducible data were obtained.

(Received November 17, 1966)

Gas chromatographic separation of catecholamines found in human or other mammalian tissues is one of the most interesting problems,[1~4] but reports dealing with them are not many and a satisfactory general procedure for their determination from biological materials has not been found. The main difficulty seems to be the extreme lability of and polyfunctional groups in the molecule of catecholamines. The objective of this study was to develop a suitable method for gas chromatographic determination of catecholamines in biological materials. Previously, we[5] reported that a satisfactory separation of the catecholamines and related compounds was achieved through trimethylsilylation with hexamethyldisilazane, using pyridine as a solvent, followed by condensation with 2-pentanone. Later, pyridine was found to be unsuitable for quantitative determination of such extremely unstable catecholamines, since it required a long duration at a higher temperature for complete trimethylsilylation. Recently, Capella and Horning[6] used dimethylformamide as a reaction medium. The trimethylsilylation of catecholamines is greatly affected by the nature of solvents used. Compared with pyridine, dimethylformamide accelerated the trimethylsilylation 10 to 20 times, which is a very important fact because it is necessary to complete the reaction in as short a time as possible to avoid the decomposition of unstable compounds such as catecholamines. However, Capella, *et al.* encountered a difficulty in that dimethylformamide itself had a tendency to condense with primary amines such as octopamine and norepinephrine. Therefore, they carried out all their procedure at a room temperature in which the reaction mixture was allowed to stand for 12 hours. As reported in our preliminary communication,[7] we found dimethyl sulfoxide to be an excellent solvent for trimethylsilylation of catecholamines; it accelerated the reaction as fast as dimethylformamide did and yet did not produce any by-product even at 80°. The method using dimethyl sulfoxide was applied to the determination of catecholamines from bovine adrenal medulla and satisfactory results were obtained.

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1) N.P. Sen, P.L. McGeer : Biochem. Biophys. Res. Commun., **13**, 390 (1963).

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Experimental

Apparatus——A Shimadzu Model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector was used, and the column were of stainless steel U-tubes, 1.5 m. in length and 4 mm. in internal diameter. A flow rate of the carrier gas (nitrogen) was 80 ml./min. The column and the injection temperatures were 170°, and the detector was kept at 180°.

Preparation of Packings——Gas-Chrom P (Applied Science Laboratories, State College, Pennsylvania), 60 to 80 mesh, treated with dimethyldichlorosilane was immersed in 7% DC 1107 siloxane polymer dissolved in anhydrous ether and ether was slowly evaporated with occasional agitations at a room temperature.

Preparation of Derivatives——The following method was developed after some preliminary studies. One ml. of a sample solution in 0.2*N* AcOH containing more than 30 μg. of each catecholamine was evaporated to dryness in a water bath of 40° at a reduced pressure. The residue was treated with 0.1 ml. of dimethyl sulfoxide, 0.1 ml. of dioxan,[*2] and 0.2 ml. of hexamethyldisilazane for 10 min. at 80°. The reaction solution was washed with cold H_2O to avoid pollution of the detector. When cooled, an aliquot of $CHCl_3$ solution containing a known amount of benzyl β-phenylpropionate (internal standard), 1 ml. each of $CHCl_3$ and cold H_2O were added to the reaction mixture, which was shaken vigorously for several seconds, and centrifuged at 1,000 to 2,000 r.p.m. for 1 min. The upper aqueous phase was pipetted off. After drying over anhydrous Na_2SO_4, the $CHCl_3$ phase was concentrated to a small volume[*3] under suction at a room temperature. Then, 0.5 ml. of 4-methyl-2-pentanone was added, the solution was allowed to stand for 30 min. at a room temperature, and was injected to a gas chromatograph.

Extraction of Catecholamines from Bovine Adrenal Medulla——The procedure was essentially the the same as that of von Euler and Floding.[8)]

After the bovines were killed, the adrenal medullae were immediately dissected out. Two g. of the organ was homogenized in a Potter glass homogenizer containing 10 ml. of 10% trichloroacetic acid. The homogenate was centrifuged in a refrigerated centrifuge for 10 min. at 10,000 r.p.m. The supernatant was decanted into a beaker and the residue was re-extracted with 5 ml. of 5% trichloroacetic acid. Two g. of Al_2O_3 was added to the combined extract and the solution was adjusted using a pH meter to about pH 7 with 4*N* NH_4OH, then to pH 8.5 with 1*N* NH_4OH with stirring by a magnetic stirrer. All the steps of extraction described above were carried out at about 0°. The supernatant was discarded and Al_2O_3 was transferred to a chromatographic tube. After rinsing with 20 ml. of distilled H_2O, the column was eluted with 20 ml. of 0.2*N* AcOH.

Fluorometry——Determination of epinephrine and norepinephrine was carried out according to the method of von Euler and Floding.[8)]

Colorimetry——Determination of epinephrine and norepinephrine was carried out according to the method of von Euler and Hamberg.[9)]

Results and Discussion

Solvents——When dimethyl sulfoxide was used as a solvent, epienephrine reacted with hexamethyldisilazane at 80° and the solution became completely transparent in 5~10 min. Norepinephrine and dopamine reacted more readily. The fact that the formation of derivatives was achieved quickly without decomposition was extremely significant and led to the successful gas chromatographic separation of catecholamines from biological materials. Tetrahydrofuran and acetonitrile were also tested as solvents, but successful results were not obtained. As a solvent for extraction, chloroform, methylene chloride, carbon tetrachloride, and *sym*-tetrachloroethane were examined and the use of chloroform as an extraction fluid seemed to be preferable to the others in respect to the reproducibility of extraction.

Schiff Base Formation——The trimethylsilylated primary amines such as dopamine and norepinephrine condensed readily with various aliphatic ketones at a room temperature to form the Schiff's bases. Several aliquot amounts of ketone were added to the chloroform solution containing a known amount of trimethylsilylated primary

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[*2] Dioxan was added to make dimethyl sulfoxide miscible with hexamethyldisilazane.

[*3] When evaporated to dryness, an appreciable decomposition of the products was observed.

8) U.S. von Euler, I. Floding: Acta Physiol. Sband., **33**, Suppl. 118 (1955).

9) U.S. von Euler, U. Hamberg: Acta Physiol. Scand., **19**, 74 (1949).

amine and the mixture was allowed to stand at a room temperature. The progress of the condensation reactions was checked by gas chromatography. Condensation with ketones seems to be an equilibrium reaction. Fig. 1 shows the effect of the amount of the ketone on condensation with an amine. When the concentration of ketone added was adequate (A), most of the condensation reaction was completed in original 15 minutes, but at a lower concentration of the ketone (B and C), the reaction rate decreased and in the case of C, even after a long duration, the peak of free amine did not disappear. Among aliphatic methyl ketones tested, acetone was the most reactive and 3-pentanone reacted incompletely, probably because of the bulkier ethyl group (Fig. 1, D). Warming did not have much noticeable effect on the condensation. Epinephrine, which is a secondary amine, was not affected by the reaction condition described in the experimental section. Cyclic and aromatic ketones were not investigated.

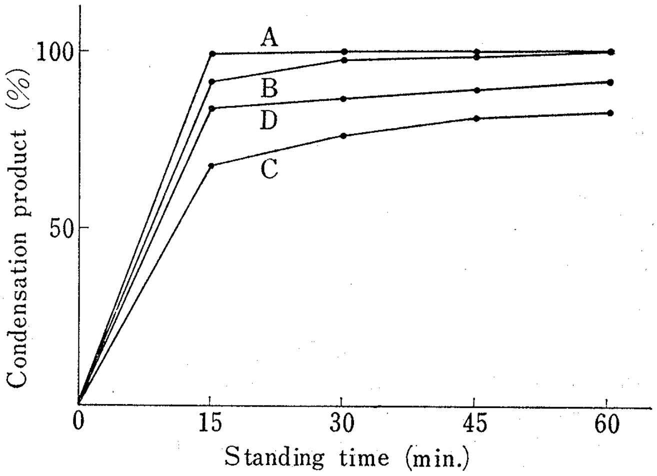

Fig. 1. Effect of the Concentration of Ketone 4-methyl-2-pentanone) on Condensation with Norepinephrine at a Room Temperature

	Norepinephrine (mg.)	4-methyl-2-pentanone (ml.)	$CHCl_3$ (ml.)
A	0.2	0.4	0.1
B	0.2	0.2	0.3
C	0.2	0.05	0.45
		3-Pentanone	
D	0.2	0.4	0.1

Separation and Estimation of Model Samples——Relative retention times for trimethylsilyl derivatives of catecholamines, related compounds, and their condensates with various kinds of ketones are given in Table I including several compounds expected as internal standards. When 4-methyl-2-pentanone was used for the condensation, benzyl β-phenylpropionate was suitable as an internal standard. When dopamine is absent in a sample, it is desirable to use acetone as a condensation reagent and allethrin as an internal standard. Fig. 2 shows calibration curves on epinephrine, dopamine, and norepinephrine using benzyl β-phenylpropionate as an internal standard. This graph

Table I. Relative Retention Times of Various Derivatives of Catecholamines and Related Compounds Including Several Compounds Expected as an Internal Standard

Compounds	Relative retention time[a]	Compounds	Relative retention time[a]
Tyramine TMSi[b]	0.27	Dopamine TMSi-3-Pentanone	1.75
Dopamine TMSi	0.60	Dopamine TMSi-2-Pentanone	1.78
Metanephrine TMSi	0.80	Norepinephrine TMSi-2-Butanone	2.05
Epinephrine TMSi	1.00	Dopamine TMSi-4-Methyl-2-pentanone	2.07
Dopamine TMSi-Acetone	1.00	Norepinephrine TMSi-2-Pentanone	2.20
Norepinephrine TMSi	1.00	Allethrin	2.40
Dopamine TMSi-2-Butanone	1.32	Norepinephrine TMSi-3-Pentanone	2.58
Benzyl β-phenylpropionate	1.33	Norepinephrine TMSi-2-Pentanone	2.62
Norepinephrine TMSi-Acetone	1.54	Norepinephrine TMSi-4-Methyl-2-pentanone	2.97

a) 7% DC 1107, 1.5 m., 170° b) TMSi: trimethylsilyl derivative shows that their quantitative analyses are possible between 30 and 120 μg. Calibration curve for epinephrine did not pass through the zero point and the reason for it might be due to its partial decomposition during the process of treatment. Table II shows the variation of the data obtained from gas chromatographic analysis of epinephrine, in which the standard deviation was about 7%.

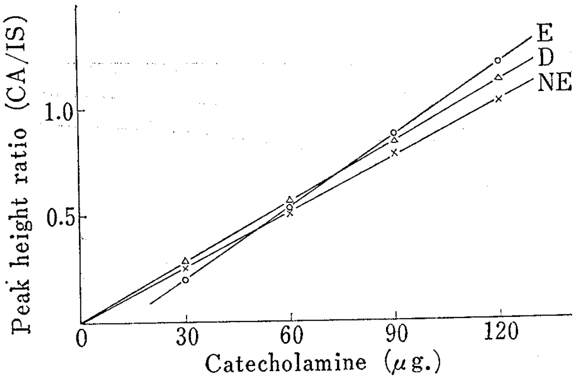

Fig. 2. Calibration Curves for Epinephrine (E), Dopamine (D), and Norepinephrine (NE) using Benzyl β-Phenylpropionate as an Internal Standard

0.3 ml., 0.6 ml., 0.9 ml., and 1.2 ml. of the model solution containing 100 μg. of each catecholamine in 1 ml. of 0.2*N* acetic acid were respectively treated by the same method as described in the experimental section.

Determination of Catecholamines from Bovine Adrenal Medulla——Each 0.5 ml. of the extract obtained from bovine adrenal medulla was analysed. A successful separation using 4-methyl-2-pentanone as a condensation reagent is illustrated in Fig. 3. In Table III, A shows the values of catecholamines obtained by this procedure.

TABLE II. Variation of the Data in Gas Chromatographic Analysis[a] on Epinephrine[b]

	Peak height of epinephrine (E) (mm.)	Peak height of allethrin[c] (A) (mm.)	E/A
1)	42.0	13.8	3.06
2)	59.0	19.8	2.99
3)	57.1	19.0	3.19
4)	44.7	15.5	2.87
5)	56.9	17.1	3.33
Average			3.09
Standard deviation			0.21

a) 7% DC 1107, 1.5 m., 170°.
b) On 1.0 ml. of 0.2*N* acetic acid containing 0.5 mg. of epinephrine.
c) Allethrin was used as an internal standard.

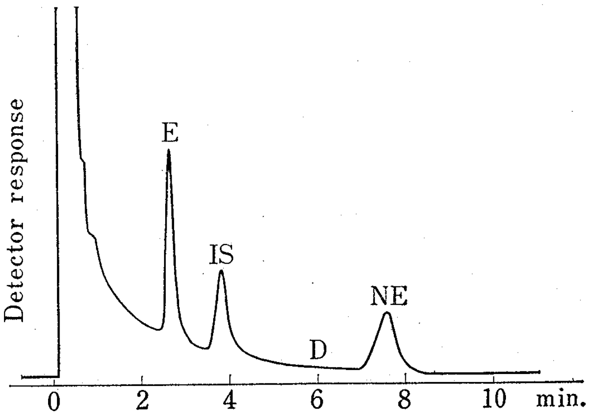

Fig. 3. Gas Chromatogram of Catecholamines from Bovine Adrenal Medulla

Trimethylsilyl derivative of epinephrine (E) and Schiff base-trimethylsilyl derivatives (4-methyl-2-pentanone condensation products) of dopamine (D) and norepinephrine (NE).
IS : Internal standard (benzyl β-phenylpropionate)
Condition : 7% DC1107 on 60/80 mesh Gas-Chrom P, 1.5 m. × 4 mm., 170°, and 80 ml. of nitrogen per min.

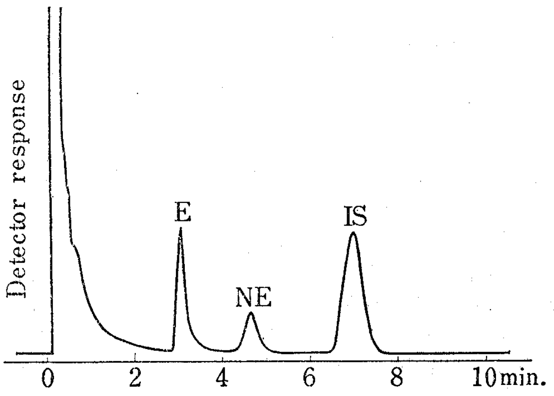

Fig. 4. Gas Chromatogram of Catecholamines from Bovine Adrenal Medulla

Trimethylsilyl derivative of epinephrine (E) and Schiff base-trimethylsilyl derivative (acetone condensation product) of norepinephrine (NE).
IS : Internal standard (allethrin)
Conditions are the same as described in Fig. 3.

As dopamine was not detected, an alternative method using acetone was tried (Fig. 4) and the values obtained are shown by B in Table III. The values in A and B seem to show a good agreement. C gives the data on additional tests in which 500 μg. of epinephrine in 0.2N acetic acid was added to 1.0 ml. of the same extract and analysed by the same method as B. An av. 1 in the first column of Table III represents the average of two values of epinephrine obtained in B and an av. 2 means that of two values obtained from the additional tests (C). Av.2−av.1 (the recovery of additional test) was 96%. These data were also compared with the values obtained from colorimetric and fluorimetric estimation of the same extract. The latter value was rather lower than that of the former, and the gas chromatographic data are fairly lower than those obtained from fluorimetric estimation. Gas chromatographic method achieves the complete separation of epinephrine and norepinephrine, so the values obtained by it seem to be most reliable and dopamine, metanephrine, and normetanephrine, if any, can be detected simultaneously.

TABLE III. Determination of Catecholamines from Bovine Adrenal Medulla

		Epinephrine (μg./ml.)	Norepinephrine (μg./ml.)	Total (μg./ml.)
A	1	276	96	372
	2	293	87	380
B	1	262	84	346
	2	272	103	375
	av. 1	267		
C	1	764	80	844
	2	730	74	804
	av. 2	747		
	av. 2−av.1	480 μg. (96%)		
Colorimetry		518	146	664
Fluorometry		430	100	530

We are grateful to Dr. T. Nagatsu for his continued support and encouragement.