CHROM. 5164

DETERMINATION OF METABOLITES OF TYROSINE AND OF TRYPTOPHAN AND RELATED COMPOUNDS BY GAS–LIQUID CHROMATOGRAPHY

PHILLIP W. ALBRO AND LAWRENCE FISHBEIN

National Institute of Environmental Health Sciences, National Institutes of Health, Public Health Service and Department of Health, Education and Welfare, Research Triangle Park, N.C. 27709 (U.S.A.)

(Received November 17th, 1970)

SUMMARY

A one-step procedure for the simultaneous derivatization of acidic, neutral and basic metabolites of tyrosine and tryptophan has been developed. The derivatives of sixteen tryptophan metabolites, twelve tyrosine metabolites and several related compounds were quantitatively determined by gas–liquid chromatography using three stationary phases of varying selectivities.

INTRODUCTION

The metabolism of tryptophan and tyrosine is becoming of increasing interest[1–5] due primarily to the varied activities of their metabolites in the nervous system, but also due to the association of these metabolites with various disorders such as Kwashiorkor[6] and malignant carcinoid[7]. Metabolites of tryptophan and of tyrosine have been determined in a variety of ways, including gas–liquid chromatography (GLC) of acidic[8] and basic[9,10] components separately.

We have developed a procedure for the GLC analysis of sixteen tryptophan metabolites, twelve phenylalanine/tyrosine metabolites, the parent amino acids and a number of related compounds. Carboxy-, amino- and polyfunctional metabolites are determined simultaneously.

MATERIALS AND METHODS

Silylating agents were obtained from Pierce Chemical Co., Rockford, Ill. Reference amino acids and metabolites were from Sigma Chemical Co., St. Louis, Mo. Gas chromatography stationary phases and solid supports were from Applied Science Laboratories, State College, Pa., and from Supelco, Inc., Bellefonte, Pa.

Analyses were performed using Varian Aerograph Model 1200 and Hewlett-Packard Model 5750 gas chromatographs, both equipped with hydrogen flame ionization detectors. Injection port and detector temperatures were 260 and 300°, re-

spectively, while in all cases the helium flow rates were initially 45 ml/min measured at the column outlet. Hydrogen and air flows were 22 and 250 ml/min, respectively. Columns were 3.1 m × 3.2 mm O.D. stainless steel; they contained the packings and were temperature-programmed as described in Table I.

Reactions were carried out in 1-ml vials equipped with Teflon-lined screw caps

TABLE I

CONDITIONS FOR GAS CHROMATOGRAPHY

System	*Liquid phase*	*Solid support*	*Linear program*
A	7% OV-1	100/120 Gas-Chrom Q	120–265° at 6°/min.
B	10% OV-17	80/100 Supelcoport	150–270° at 6°/min.
C	10% OV-210	80/100 Supelcoport	150–245° at 6°/min.

(Reactivials, Pierce Chemical Co.). 3-μl samples of the reaction mixtures were injected directly into the gas chromatographs.

Studies with model compounds

Indole, hydroxyindole, 2-phenyl-ethylamine, indole-3-acetic acid, 5-hydroxytryptophan and 5-hydroxyindole-3-acetic acid were individually studied to compare the derivatizations of the various functional groups by different silylating reagents. Solutions or suspensions of the starting materials in pyridine were chromatographed to determine the retention times of those unreacted compounds capable of eluting.

Silylation reactions were run either at room temperature for 3 h, at 100° for 10 min, or at 100° for 10 min followed by overnight standing at room temperature. 1-mg samples of each compound to be derivatized were reacted with 50–300 μl quantities of: (1) trimethylsilylimidazole (TMSI), (2) N-trimethylsilyl diethylamine (TMSDEA), (3) hexamethyldisilazane–trimethylchlorosilane–pyridine (HMDS–TMCS–PYR), 10:5:25 (v/v/v), (4) HMDS–TMCS–Dimethylformamide, 10:5:25 (v/v/v), (5) bis-trimethylsilyltrifluoroacetamide (b-TMSTFA) containing 1% TMCS, (6) b-TMSTFA–TMCS–PYR, 99:1:250 (v/v/v), (7) TMSDEA–TMSI, 1:1 (v/v), (8) 30% TMSDEA in pyridine, (9) 30% TMSI in pyridine, (10) TMSDEA–TMCS, 8:1 (v/v), (11) b-TMSTFA–TMSDEA–TMCS–PYR, 99:30:1:100 (v/v/v/v).

Quantitative measurements

Peak areas were measured as height × width at half-height, and were expressed relative to the peak area of an internal standard, usually *n*-docosane. These measurements were used to determine relative molar response factors for the various compounds under the conditions used. It was found that the same relative molar response factors applied whether the Varian or the Hewlett-Packard gas chromatographs were used.

Assumptions

It was assumed that any silylating procedure that did not produce a silylated derivative with indole would not react with the indole nitrogen of tryptophan metabolites. Similarly, failure to derivatize phenylethylamine was assumed to indicate

that the reagent would not derivatize amino groups in the other test substances. These assumptions were used only to eliminate reagents from further consideration; therefore, the final results presented below do not depend upon the assumptions being valid.

Recommended procedures

Add *n*-docosane to reagent (11) above to give 50 μg/ml. Up to 2 mg of tryptophan and/or tyrosine metabolites may be derivatized in a sealed tube with 0.30 ml of reagent, preferably by heating at 100° for 10 min followed by letting stand overnight at room temperature. The resulting solution may be analyzed directly, chromatographing 3-μl aliquots on each of the columns described in Table I.

Application to biological materials

Up to 50 mg of lyophilized, finely pulverized tissue, bacterial cells, or lyophilized urine may be reacted directly with 1 ml of reagent as described above. Insufficient reagent usually results in the formation of a gel, in which case additional reagent may be added. It was found advisable to centrifuge these preparations prior to chromatography to avoid fouling the injection block. After complete silylation, all of the compounds discussed below were found to be soluble in the reagent. A somewhat similar procedure has been previously applied to dried urine for screening purposes[11].

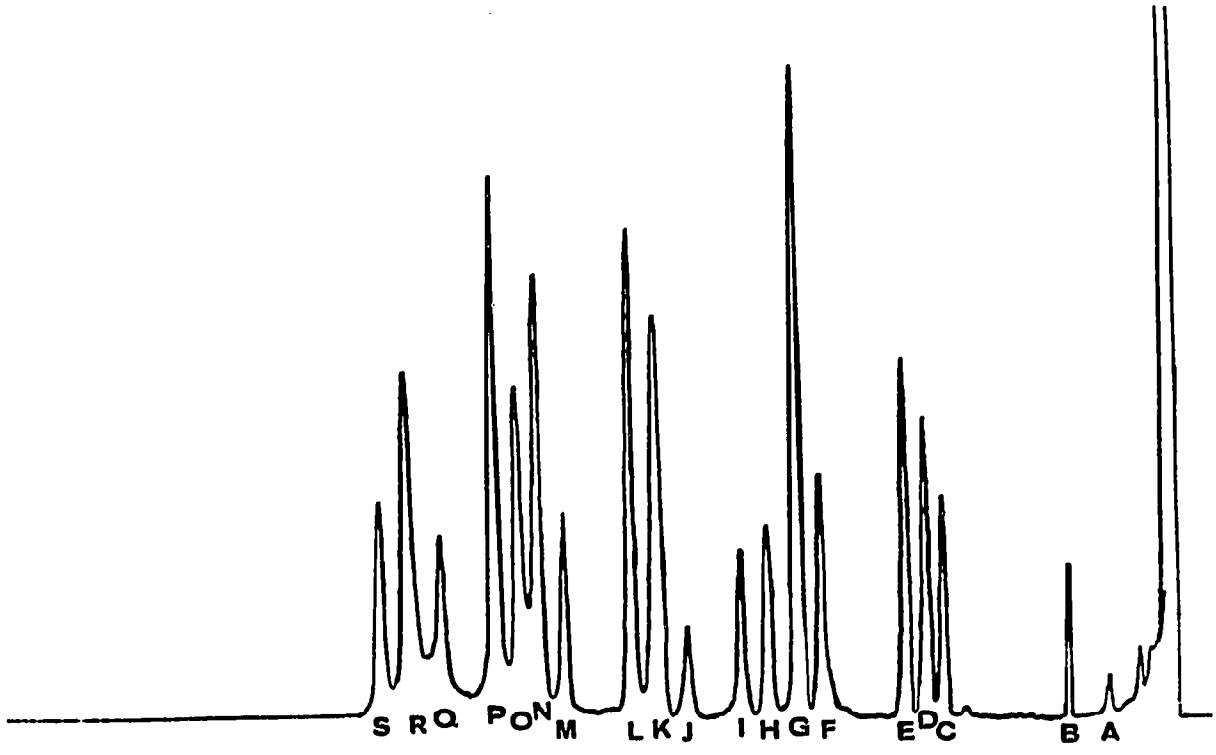

Fig. 1. Chromatography of silylated derivatives on OV-17. Identification of peaks: A = urea; B = nicotinic acid; C = nicotinamide; D = phenylalanine; E = *p*-hydroxyphenylacetic acid; F = 3-hydroxyanthranilic acid; G = homogentisic, homovanilic, and homoprotocatechuic acids; H = artifact; I = tyrosine; J = dopamine, K = Dopa plus *p*-hydroxyphenylpyruvic acid; L = indole-3-acetic acid; M = kynurenic acid; N = kynurenine; O = tryptophan plus serotonin; P = 5-hydroxyindole-3-acetic acid; Q = 3-hydroxykynurenine; R = 5-hydroxy-tryptophan; S = indole-3-pyruvic acid. For chromatographic conditions, see Table I.

RESULTS AND DISCUSSION

Model compounds

Reagents (1), (7) and (9) which contained TMSI gave multiple products with all of the model compounds. Reagents (3) and (4) gave two peaks with hydroxyindoleacetic acid when the products were chromatographed in the B system. Reagents (2) and (8) containing TMSDEA alone never gave quantitative silylation, possibly because the diethylamine produced was not removed from the reaction mixtures[12]. Reagents (5) and (6) generally gave quantitative results with the model compounds, but the derivatives of phenylethylamine partially decomposed on standing.

TABLE II

ELUTION AND RESPONSE PARAMETERS OF SILYLATED DERIVATIVES

Compound	*OV-1*[a]		*OV-17*		*OV-210*		*Rel. molar resp.*[b]
	R'_T *(min)*	E_T *(°C)*	R'_T *(min)*	E_T *(°C)*	R'_T *(min)*	E_T *(°C)*	
Urea	1.60	130.6	1.93	164.6	2.20	166.2	—
Nicotinic acid	4.04	145.2	2.62	168.7	3.37	173.4	0.058
Indole	6.30	158.8	5.05	180.3	4.48	179.8	0.117
Nicotinamide	6.96	162.8	5.80	187.8	7.88	200.6	0.093
Creatinine	9.56	178.4	4.73	181.4	4.66	181.0	—
Phenyllactic acid	9.62	178.7	5.74	187.4	5.02	183.1	0.174
Phenylalanine	10.40	183.4	5.90	188.4	5.75	187.5	0.196
p-Hydroxyphenylacetic acid	10.41	183.5	6.88	194.3	6.62	192.7	0.157
Tryamine	10.42	183.5	5.70	187.2	5.09	183.5	0.153
5-Hydroxyindole	12.75	197.5	10.00	213.0	9.00	207.0	0.152
Homovanilic acid	12.78	197.6	9.51	210.0	9.18	208.1	0.200
3-Hydroxyanthranilic acid	12.91	198.5	8.90	206.4	8.79	206.1	0.277
Homoprotocatechuic acid	14.37	207.2	9.51	210.0	9.25	208.9	0.546
Homogentisic acid	14.44	207.7	9.53	210.2	9.85	212.5	0.546
4-Pyridoxic acid	16.30	218.9	11.61	222.7	10.90	218.4	0.580
Indole-3-acetic acid	16.31	219.0	13.59	243.5	13.54	234.9	0.399
Tyrosine	16.40	219.4	10.60	216.6	10.63	216.8	0.213
L-Epinephrine	17.70	227.2	10.00	213.0	8.00	201.0	0.682
p-Hydroxyphenylpyruvic acid	18.38	231.3	12.90	230.4	12.50	228.0	0.206
Dopamine	19.19	236.1	12.15	225.9	11.22	221.0	0.438
Uric acid	19.20	236.2	14.52	240.1	11.04	219.0	—
Dopa	19.50	238.0	12.99	230.9	12.91	231.1	0.446
Tryptamine	19.83	240.1	12.37	220.5	11.62	222.8	0.068
Kynurenic acid	20.31	243.0	15.29	244.7	15.48	245	0.103
n-Docosane	20.61	244.8	13.67	235.0	10.71	217.4	1.000
Kynurenine	20.64	245.0	15.98	248.9	15.50	245	0.193
5-Methoxyindole-3-acetic acid	20.65	245.0	17.52	258.1	17.79	245	0.431
Xanthurenic acid	20.80	245.8	16.98	254.9	16.50	245	0.456
Serotonin	20.88	246.2	16.25	250.5	15.85	245	0.078
5-Hydroxyindole-3-acetic acid	20.90	246.4	16.98	254.9	16.93	245	0.467
Tryptophan	21.10	247.6	16.40	251.4	16.58	245	0.139
Indole-3-pyruvic acid	24.25	265	19.80	270	20.40	245	0.208
3-Hydroxykynurenine	24.37	265	18.32	262.9	18.38	245	0.206
5-Hydroxytryptophan	24.95	265	19.10	267.6	19.60	245	0.149
N-Acetyl-5-serotonin	25.23	265	23.60	270	24.01	245	0.129

[a] R'_T (min) = corrected elution time in minutes; E_T (°C) = temperature at peak elution.
[b] Molar response relative to *n*-docosane in terms of peak areas.

Reagents (10) and (11) were quite satisfactory for quantitative conversion of the model compounds into gas chromatographable derivatives. They differed in that reagent (10), TMSDEA–TMCS, 8:1, did not react with indole while reagent (11), b-TMSTFA–TMCS–TMSDEA–PYR, 99:1:30:100, did; thus the two reagents gave different derivatives with the various tryptophan metabolites. We were unable to find chromatographic conditions that would resolve all of those tryptophan metabolites derivatized by reagent (10); therefore, reagent (11) was finally selected as most suitable for our purposes.

Complex mixtures of tryptophan and tyrosine metabolites

Fig. 1 shows a typical chromatogram obtained with the OV-17 column in the Varian Aerograph chromatograph. This column did not separate the derivatives of tryptophan and serotonin, of Dopa and *p*-hydroxyphenylpyruvic acid, and of homovanilic, homogentisic, and homoprotocatechuic acids. These derivatives were, however, resolved on OV-210. OV-210 did not resolve hydroxyindole from homovanilic acid, while this separation could be accomplished on either OV-1 or OV-17. The dopamine–tryptamine pair was best resolved on OV-1.

Table II lists the compounds tested, their elution parameters for the three sets of conditions used, and their molar responses relative to that of *n*-docosane. It is clear from Table II that separation of all the compounds listed requires the use of all three columns.

Analysis of "spiked" urine

A water solution containing indole, dopamine, kynurenic acid, hydroxyindoleacetic acid, hydroxykynurenine, and N-acetylserotonin was prepared, representing various functional groups to be expected in biological materials. 1 ml of standard mixture was diluted with 1 ml of water, 1 ml of standard was mixed with 1 ml of rat urine, and 1 ml of rat urine was diluted with 1 ml of water after which the three solutions were freeze-dried and analyzed as described above. The ratios of the peak areas of the "spike" compounds to that of the internal standard (*n*-docosane) were compared as shown in Table III.

TABLE III

ANALYSIS OF SPIKED URINE

Compound	*Peak area ratios*[a]			
	Spike	*Control urine*	*Spiked urine*	
			Calc.[b]	*Found*
Indole	0.49	0.53	1.02	0.95
Dopamine	1.41	0.25	1.66	1.65
Kynurenic acid	0.06	0.08	0.14	0.15
Hydroxyindoleacetic acid	2.20	0.13	2.33	2.32
Hydroxykynurenine	0.04	0.11	0.15	0.13
N-Acetyl serotonin	0.79	0.15	0.94	1.01

[a] Peak area as fraction of *n*-docosane (internal standard) peak area.
[b] Sum of spike + control urine.

It is clear from Table III that the constituents of normal rat urine do not interfere with determination of the test metabolites. An unusually high amount of urea will interfere as has been indicated previously[11], but we found that a urea content high enough to exhaust the reagent inevitably resulted in formation of an obvious gel. In such a case it was necessary to add additional reagent and warm briefly.

REFERENCES

1 D. K. ZHELYASKOV, M. LEVITT AND S. UDENFRIEND, *Mol. Pharmacol.*, 4 (1968) 445.
2 H. VARLEY AND A. H. GOWENLOCK (Editors), *West-European Symposia on Clinical Chemistry*, Vol. 2. Elsevier, New York, 1963.
3 J. JACOB, *Proc. Eur. Soc. Study Drug Toxicity*, 8 (1967) 59.
4 J. J. SCHILDKRAUT AND S. S. KETY, *Science*, 156 (1967) 21.
5 C. L. GAZZULLO, A. MANGONI AND G. MASCHERPA, *Brit. J. Psychiat.*, 112 (1966) 157.
6 A. S. ABBASSY, M. M. ZEITOUN, E. A. HASSANEIN, G. A. ABDEL-TAWAB, T. S. KHOLIEF AND S. W. EL-SEWEDY, *Trans. Roy. Soc. Trop. Med. Hyg.*, 64 (1970) 27.
7 G. T. BRYAN, *Ann. Ind. Hyg. Ass. J.*, 30 (1969) 27.
8 M. G. HORNING, K. L. KNOX, C. E. DALGLIESH AND E. C. HORNING, *Anal. Biochem.*, 17 (1966) 244.
9 C. J. W. BROOKS AND E. C. HORNING, *Anal. Chem.*, 36 (1964) 1540.
10 E. C. HORNING, M. G. HORNING, W. J. A. VANDENHEUVEL, K. L. KNOX, B. HOLMSTEDT AND C. J. W. BROOKS, *Anal. Chem.*, 36 (1964) 1546.
11 R. F. COWARD AND P. SMITH, *J. Chromatog.*, 37 (1968) 111.
12 P. S. MASON AND E. D. SMITH, *J. Gas Chromatog.*, 4 (1966) 398.