ANALYSIS OF N-METHYLAMINO ACIDS BY AUTOMATIC AMINO ACID ANALYZER AND GAS-LIQUID CHROMATOGRAPHY

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INTRODUCTION

N$^{\alpha}$-Monomethylated L-amino acids have been found in a series of peptide antibiotics such as Actinomycins, Etamycin and the quinoxaline antibiotics which have been discovered and elucidated during the last few years. They are N-methyl derivatives of L-alanine[1], L-cystine[2], glycine (sarcosine)[1], L-isoleucine[1,3,4], L-alloisoleucine[5], L-leucine[6], L-phenylalanine[7], L-phenylglycine (α-phenylsarcosine)[8] and γ-methyl-L-alloisoleucine[8,9]. N-Methyl-D-tyrosine (D-surinamine)[10] and N-methyl-L-tryptophan (L-abrin)[11-13] have been found as the products of plants. It therefore cannot be doubted that N-methylamino acids play a very important role as constituents of peptide or depsipeptide antibiotics.

However, there was no simple or generally applicable method for the analysis of these N-methylamino acids. Most of the reactions for the detection of secondary amines are not suitable for quantitative analysis of N-methylamino acids.

In the present paper, the authors have shown that the ninhydrin developing method used by the automatic amino acid analyzer or determination by gas-liquid chromatography could be useful for the analysis of N-methylamino acids, although some limitations were found. As an example, a series of quinoxaline antibiotics which contained some N-methylamino acids were analyzed by both methods.

EXPERIMENTAL

Preparation of N-methylamino acids

N-Methylated derivatives of L-alanine, L-arginine, L-aspartic acid, L-cysteic acid, L-cystine, L-glutamic acid, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-serine, L-threonine, L-tyrosine and L-valine were prepared by the method of QUITT *et al.*[14]. A detailed description of the synthesis of these N-methylamino acids appears in a previous paper[15]. Sarcosine was purchased from Kanto Chemical Co. Ltd., Osaka, N-methyl-D,L-alloisoleucine and N,γ-dimethyl-D,L-alloisoleucine were supplied by Dr. JUNICHI SHOJI. Melting points, optical rotations and elementary analyses of these compounds are shown in Table I.

Quinoxaline antibiotics

Quinomycins A, B, B_0, C, D, E and Triostin A and C were supplied by Dr. J. SHOJI. For the analysis of these antibiotics, an aliquot of each pure compound was

TABLE I

MELTING POINTS, OPTICAL ROTATIONS AND ELEMENTARY ANALYSES OF N-METHYLAMINO ACIDS

N-Methylamino acid	*Formula*	*Molecular weight*	*M.p.*	$[\alpha]_D^{25}$
N-Methyl-L-alanine	$C_4H_9O_2N$	103.12	260–263°	+10.4 c = 1, 6 N H
Nα-Methyl-L-arginine	$C_7H_{16}O_2N_4$	188.23	260°	+32.2 ,, ,,
N-Methyl-L-aspartic acid	$C_5H_9O_4N \cdot {}^1/_2H_2O$	156.14	168°	+25.3 ,, ,,
N-Methyl-L-cysteic acid	$C_4H_9O_5NS$	183.18	219–220°	+ 6.7 ,, ,,
N,N′-Dimethyl-L-cystine	$C_8H_{16}O_4N_2S_2 \cdot {}^1/_2H_2O$	277.36	206–208° dec.	+98.8 ,, ,,
N-Methyl-L-glutamic acid	$C_6H_{11}O_4N \cdot {}^3/_4H_2O$	174.67	131°	+30.6 c = 2, 6 N H
N-Methyl-glycine (sarcosine)	$C_3H_7O_2N$	89.09	210–215° dec.	—
N-Methyl-L-isoleucine	$C_7H_{15}O_2N$	145.20	240°	+46.1 c = 0.5, 6 N H
N-Methyl-D,L-alloisoleucine	$C_7H_{15}O_2N$	145.20	—	—
N-Methyl-L-leucine	$C_7H_{15}O_2N$	145.20	220° sub.	+30.0 c = 1, 6 N H
Nα-Methyl-L-lysine (HCl)	$C_7H_{16}O_2N_2 \cdot HCl$	196.68	234–235°	+28.5 ,, ,,
N-Methyl-L-phenylalanine	$C_{10}H_{13}O_2N$	179.22	233–237° dec.	+26.6 ,, ,,
N-Methyl-L-serine	$C_4H_9O_3N$	119.12	194°	+ 9.8 ,, ,,
N-Methyl-L-threonine	$C_5H_{11}O_3N$	133.15	240°	−15.9 ,, ,,
N-Methyl-L-tyrosine (L-surinamine)	$C_{10}H_{13}O_3N \cdot {}^1/_8H_2O$	197.47	149°	+29.3 c = 1, 6 N H acetic acid = 1:1
N-Methyl-L-valine	$C_6H_{13}O_2N$	131.18	>300°	+29.3 c = 1, 6 N H

hydrolyzed with three times glass-distilled 5.9 N HCl for 20 h at 110° in an evacuated and sealed tube. The hydrolysates were evaporated to dryness *in vacuo* at room temperature, and then analyzed.

Analysis of N-methylamino acids by automatic amino acid analyzer

A sample which contained 2 to 10 μmoles of each N-methylamino acid or a hydrolysate of a quinoxaline antibiotic was applied to a column of the Hitachi KLA type 2 amino acid analyzer. Column length was selected as specified in the Tables. The resin was Amberlite CG 120, type III. Combinations with column length and buffers used were as follows: Buffers of 0.2 M sodium citrate, pH 3.25 and pH 4.25 were used for analysis with 150-cm columns; buffers of 0.2 M sodium citrate, pH 3.25, pH 4.25 and 0.7 M sodium citrate, pH 5.28, were used for 50-cm columns; and 0.35 M sodium citrate, pH 5.28, was used for 15-cm columns. Most of the other procedures followed were according to the directions of SPACKMAN *et al.*[16].

Analysis of N-methylamino acids by gas–liquid chromatography

To approximately each 1 μmole of N-methylamino acids or hydrolysate of the quinoxaline antibiotics was added 5 ml of absolute methanol and 1.00 μmole of L-leucine methyl ester hydrochloride as a standard calibration material. The mixture was saturated with anhydrous hydrogen chloride at 0° and then heated at 80° for 60 min. The esterified sample was dried at room temperature under reduced pressure, and then treated with 1 ml of trifluoroacetic anhydride for 15 min at 50°, whilst protecting it from moisture. Excess trifluoroacetic anhydride and trifluoroacetic acid were evaporated off with a stream of dry nitrogen and the methyl esters obtained were redissolved in a small amount of acetone for chromatography.

Analysis									
Calculated					*Found*				
C	*H*	*N*	*Cl*	*S*	*C*	*H*	*N*	*Cl*	*S*
46.59	8.80	13.58	—	—	46.52	9.00	13.70	—	—
44.67	8.57	29.77	—	—	44.08	8.67	29.11	—	—
38.46	6.46	8.97	—	—	37.55	6.87	9.02	—	—
26.23	4.95	7.65	—	17.50	26.00	5.32	7.62	—	16.02
34.64	6.18	10.10	—	23.12	34.67	6.37	10.02	—	22.65
41.26	7.12	8.02	—	—	41.03	7.58	8.10	—	—
40.44	7.92	15.72	—	—	40.04	7.97	15.57	—	—
57.90	10.41	9.65	—	—	57.76	10.53	9.35	—	—
57.90	10.41	9.65	—	—					
57.90	10.41	9.65	—	—	58.10	10.54	9.80	—	—
42.75	8.71	14.24	18.08	—	42.49	8.97	13.80	17.66	—
67.02	7.31	7.82	—	—	67.02	7.59	7.73	—	—
40.33	7.62	11.76	—	—	40.19	7.56	11.78	—	—
45.10	8.33	10.52	—	—	45.11	8.41	10.36	—	—
60.82	6.76	7.09	—	—	60.22	6.85	7.11	—	—
54.94	9.99	10.68	—	—	54.98	9.99	10.74	—	—

The gas–liquid chromatography instrument was a Shimazu GC type IB with an automatic temperature program controller and a hydrogen-flame detector. The column was a 4 mm I.D. × 3 m stainless steel tube containing as packing 80 to 100 mesh Chromosorb W coated with a 1% w/w copolymer of ethylene glycol succinate–siloxane (EGSS-X). The packed column was conditioned further at 210° with a nitrogen flow of 20 ml/min overnight. The column heater jacket of the gas chromatograph was allowed to equilibrate at 100°. A 1–5 μl aliquot of the solution of the amino acid derivatives (0.03–0.3 μmole of each amino acid) was injected into the flash-heater held at 210°. A nitrogen flow rate of 20 ml/min was maintained. After 20 or 22 min a programmed temperature increase of 2°/min was initiated. A total of 45 min was required for the complete analysis. Other details of the conditions are described in the text and Tables.

RESULTS

Analysis of N-methylamino acids by automatic amino acid analyzer

The positions of 17 N-methylamino acids and closely related compounds are shown in Fig. 1, with reference to the parent amino acids. The position of peaks as they appeared is shown by arrows. The position of each compound was established by individual analysis, and its relationship to other compounds by analysis of the mixture. The peaks for N-methylamino acids, in general, had good symmetry, except in some cases, *e.g.*, N-methyl derivative of isoleucine, alloisoleucine, leucine, valine, cystine and N,γ-dimethyl-alloisoleucine. Therefore, the integration method by addition of absorption readings (the fraction collector method)[16] was employed to determine these compounds. To determine the other compounds, the common

$H \times W$ method[16] was used. As shown in Fig. 1, the conversion of an L-amino acid to the corresponding N-methylamino acid is accompanied by a shift in the elution order.

In the chromatogram there was a crowded region in the neighborhood of N-methyl-aspartic acid, where some peaks have been elevated for clarity of presentation. To resolve these peaks, the buffer can be replaced by a pH 2.90 buffer instead of the buffer at pH 3.25. However, as far as the analysis of natural products, such as peptide antibiotics, is concerned, this replacement may not be necessary in order to throw light on their N-methylamino acid composition.

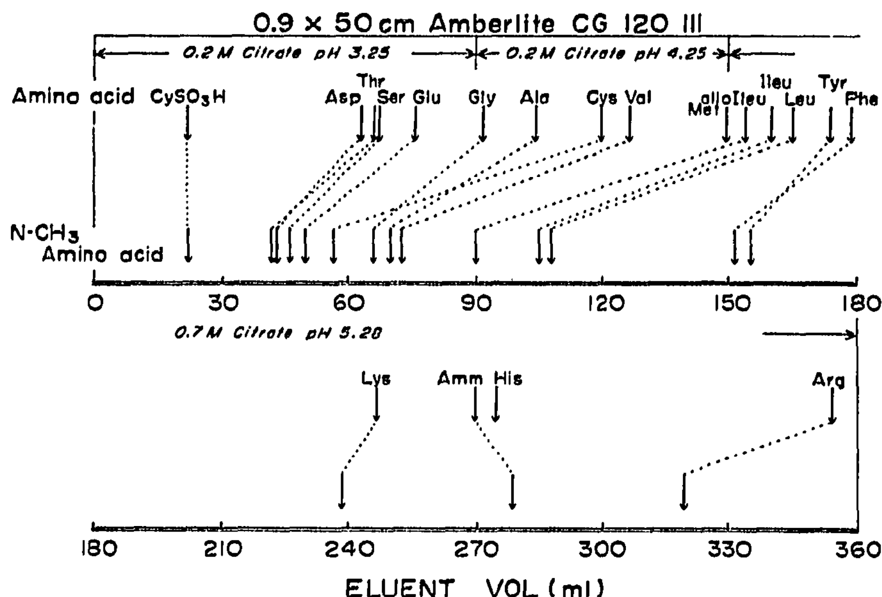

Fig. 1. The position of 17 N-methylamino acids on the 50 cm chromatogram. Arrows indicate their position as they appeared on the recording chart. Flow rate of the buffer solutions was 30 ml/h. Temperature was 50°. Buffers and the position of their changes are shown in the figure.

Table II gives the constants thus obtained for 16 N-methylamino acids and 3 closely related compounds. The list included color yield, its ratio to the parent amino acid, maximum deviation from mean and absorption ratios. The color yield of N-methylamino acids with ninhydrin was poor compared to the parent amino acids except in the case of N-methyl-L-lysine and methylamine. The maximum deviation from the average value was estimated from 2–4 analyses on an individual N-methyl-amino acid. The values obtained were somewhat higher than those of the usual amino acids.

Analysis of quinoxaline antibiotics by automatic amino acid analyzer

N-Methyl-valine, N-methyl-alloisoleucine and N,γ-dimethyl-alloisoleucine in a series of Quinomycins and Triostins were determined by an automatic amino acid analyzer. Estimation of the individual amino acids was performed after comparison with the corresponding standard run. The results obtained are listed in Table III. For a single analysis 2–5 μmoles of the antibiotic (corresponding to 2–5 mg) were required. Although the reaction between the N-methylamino acid and ninhydrin is incomplete, the recovery appeared satisfactory; this is discussed later.

TABLE II

THE COLOR YIELD OF N-METHYLAMINO ACIDS AFTER REACTION WITH NINHYDRIN

Column diameter, 0.9 cm; resin, Amberlite CG 120, III; column temperature, 50°; buffers, as described in the experimental section; flow rate, 30 ml/h.

N-Methylamino acid	*Color yield*		*Maximum deviation from mean (%)*	*Absorption ratio*		*Column length (cm)*
	C^* *in* $\frac{H \times W}{c}$	*Ratio***		$\frac{650\ m\mu}{570\ m\mu}$	$\frac{440\ m\mu}{570\ m\mu}$	
N-Methyl-L-alanine	0.982	0.081	6	0.456	0.305	50
Nα-Methyl-L-arginine	2.40	0.189	5	0.447	0.302	50
N-Methyl-L-aspartic acid	0.405	0.039	7	0.642	0.537	50
N-Methyl-L-cysteic acid	1.02	0.083	6	0.441	0.295	150 (pH 3.25)
	0.874	0.072	6	0.451	0.275	150 (pH 2.91)
N,N′-Dimethyl-L-cystine	1.52	0.125	5	0.669	0.449	150
N-Methyl-L-glutamic acid	0.637	0.066	6	0.446	0.962	50
N-Methyl-glycine (sarcosine)	2.32	0.190	5	0.432	0.314	50
N-Methyl-L-isoleucine	0.173	0.014	9	0.446	0.300	150
N-Methyl-D,L-alloisoleucine	0.171	0.014	9	0.420	0.326	150
N-Methyl-L-leucine	0.822	0.064	6	0.476	0.329	150
Nα-Methyl-L-lysine (HCl)	12.2	0.877	4	0.434	0.385	15
N-Methyl-L-phenylalanine	2.48	0.203	5	0.452	0.364	50
N-Methyl-L-serine	0.574	0.045	6	0.683	0.297	50
N-Methyl-L-threonine	0.640	0.065	6	0.455	0.298	50
N-Methyl-L-tyrosine	2.34	0.186	5	0.457	0.317	50
N-Methyl-L-valine	0.234	0.186	9	0.467	0.322	150
Methylamine	9.66	0.767	8	0.440	0.275	15
N,γ-Dimethyl-D,L-alloisoleucine	0.360	0.030	9	0.450	0.308	150
L-Alanine	11.8	1.000	3	0.448	0.273	150

* C is a constant; c is concentration. These constants are smaller than those obtained with the Beckman analyzer, since the chart width (full scale) is 15 cm.

** These values are expressed as follows:

$$\text{Ratio} = \frac{C \text{ in } \frac{H \times W}{c} \text{ of N-methylamino acid}}{C \text{ in } \frac{H \times W}{c} \text{ of parent amino acid}}$$

The absorption ratio was estimated based on the C constants at each wave length.

As an example of the analysis of the quinoxaline antibiotics, Figs. 2 and 3 show chromatograms of the hydrolysate of Triostin A and Triostin C, respectively, with a 150-cm column and 0.2 M citrate buffer, pH 3.25. Since the shape of the peak of these N-methylamino acids was not good, the so-called fraction collector method was employed to determine these compounds as described above.

Amounts found are shown in the figures as the molar content in comparison to the theoretical value. The significant losses observed with serine were due to the conditions of hydrolysis.

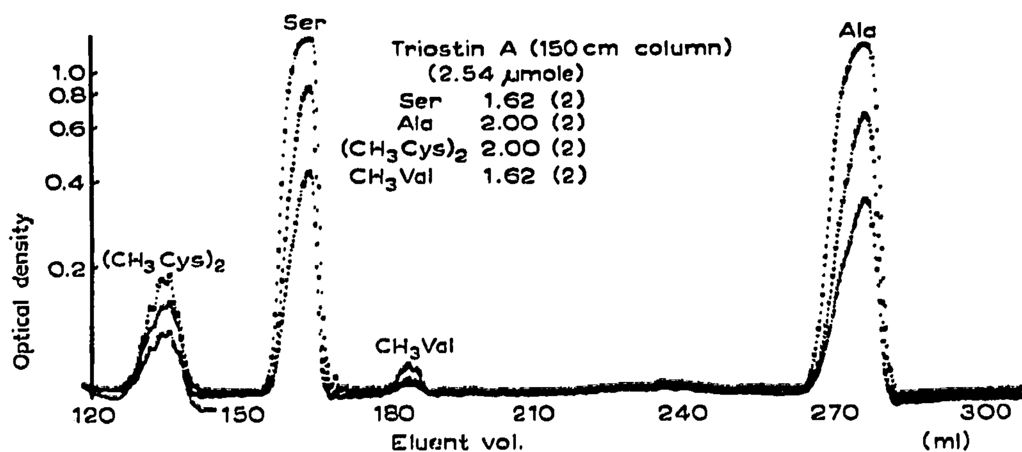

Fig. 2. Chromatogram of a hydrolysate of 2.54 μmole of Triostin A. Column, 0.9 × 150 cm; resin, Amberlite CG 120, III; temperature, 50°; buffer, 0.2 *M* citrate buffer, pH 3.25; flow rate, 30 ml/h. $(CH_3Cys)_2$ = N,N′-dimethyl-cystine; $CH_3 \cdot Val$ = N-methyl-valine. Molar content for each mole of sample is given and compared to the theoretical molar content, given in parentheses.

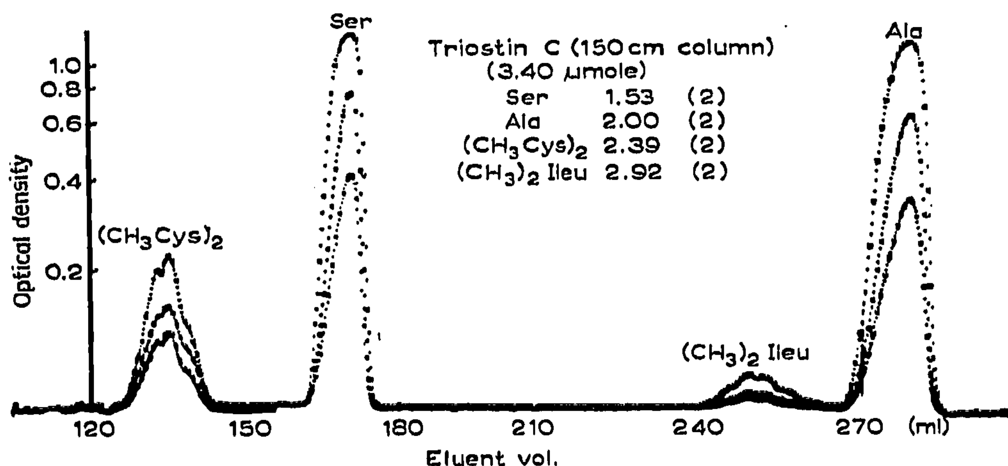

Fig. 3. Chromatogram of a hydrolysate of 3.40 μmole of Triostin C. $(CH_3)_2Ileu$ = N,γ-dimethyl-alloisoleucine. The conditions are as described in Fig. 2.

TABLE III

ANALYSIS OF SOME N-METHYLAMINO ACIDS IN QUINOXALINE ANTIBIOTICS BY AMINO ACID ANALYZER

Amounts of N-methylamino acid found are given without any correction for destruction during acid hydrolysis or the presence of impurities. The molar content of N-methylamino acid was calculated on the basis of the amount (μmole) of the sample before hydrolysis. Theoretical molar content is given in parentheses.

Quinoxaline antibiotic	*Sample amount (μmole)*	*N-Methylamino acid found*	*Amount of N-methyl-amino acid found (μmole)*	*Recovery (%)*	*Molar content in each mole of sample*	
					Found	*(Theoretical)*
Quinomycin A	2.727	N-methyl-valine	5.30	97	1.94	(2)
Quinomycin B	2.548	N-methyl-alloisoleucine	4.78	94	1.88	(2)
Quinomycin B_0	5.377	N-methyl-valine	5.59	104	1.04	(1)
		N,γ-dimethyl-alloisoleucine	5.36	100	1.00	(1)
Quinomycin C	2.439	N,γ-dimethyl-alloisoleucine	4.62	95	1.89	(2)
Quinomycin D	2.111	N-methyl-valine	2.10	99	0.99	(1)
		N-methyl-alloisoleucine	1.61	76	0.76	(1)
Quinomycin E	5.302	N-methyl-alloisoleucine	4.64	88	0.88	(1)
		N,γ-dimethyl-alloisoleucine	4.60	87	0.87	(1)
Triostin C	2.341	N,γ-dimethyl-alloisoleucine	3.75	80	1.60	(2)

Analysis of N-methylamino acids in quinoxaline antibiotics by gas–liquid chromatography

For the analysis of the quinoxaline antibiotics, a mixture of N-methyl-L-valine, N-methyl-D,L-alloisoleucine, N,γ-dimethyl-D,L-alloisoleucine, L-leucine (calibration standard material), L-alanine and L-serine were converted to the N-trifluoroacetyl-N-methylamino acid methyl esters and N-trifluoroacetylamino acid methyl esters by esterification and acylation procedures. The chromatography was carried out under the optimum conditions described in the experimental section. The results obtained are listed in Table IV. Under the conditions used no loss of resolution of any compound was found. Retention times for each amino acid derivative were very reproducible. The absolute peak areas for most of the amino acid derivatives remained remarkably constant during six to eight repetitions of the procedure with a standard mixture of amino acids.

TABLE IV

RETENTION TIMES AND PEAK AREAS FOR SOME TRIFLUOROACETYLATED N-METHYLAMINO ACID METHYL ESTERS

Peak area was estimated from the recording chart by the common $H \times W$ method. The values appearing in the Table were obtained from the calibration curve of each compound. After isothermal analysis at 100° for 22 min, the temperature was raised to 210° at a rate of 2°/min. Other conditions are as described in the experimental section.

Compound	*Temperature (°)*	*Retention time (min)*	*Peak area ($cm^2/\mu mole$)*
N-Methyl-L-valine	100	7.0	106
N-Methyl-D,L-alloisoleucine	100	10.3	110
N,γ-Dimethyl-D,L-alloisoleucine	100	12.7	120
L-Leucine	100	15.6	117
L-Alanine	100	8.2	35.3
L-Serine	114	28.6	70.5

TABLE V

ANALYSIS OF SOME N-METHYLAMINO ACIDS IN QUINOXALINE ANTIBIOTICS BY GAS–LIQUID CHROMATOGRAPHY

The chromatography was carried out under the same conditions as those described in Table IV. Calculations of molar content were performed as described in Table III.

Quinoxaline antibiotic	*Sample amount (µmole)*	*N-Methylamino acid found*	*Amount of N-methyl-amino acid found (µmole)*	*Recovery (%)*	*Molar content in each mole of sample*	
					Found	*(Theoretical)*
Quinomycin A	0.1170	N-methyl-valine	0.1360	58	1.16	(2)
Quinomycin B	0.0974	N-methyl-alloisoleucine	0.1250	64	1.28	(2)
Quinomycin B_0	0.0161	N-methyl-valine	0.0107	66	0.66	(1)
		N,γ-dimethyl-alloisoleucine	0.0111	69	0.69	(1)
Quinomycin D	0.0175	N-methyl-valine	0.0129	74	0.74	(1)
		N-methyl-alloisoleucine	0.0121	69	0.69	(1)
Quinomycin E	0.0364	N-methyl-alloisoleucine	0.0269	74	0.74	(1)
		N,γ-dimethyl-alloisoleucine	0.0270	74	0.74	(1)
Triostin C	0.358	N,γ-dimethyl-alloisoleucine	0.0387	54	1.08	(2)

The usefulness of this method of N-methylamino acid analysis was demonstrated for hydrolysates of the quinoxaline antibiotics in Table V and Fig. 4. For one analysis, very small amounts (less than 1 µmole) of the antibiotic (corresponding to less than 1 mg) were used. The calculated recoveries were somewhat lower when compared to those obtained from the analyses by the automatic analyzer as described above.

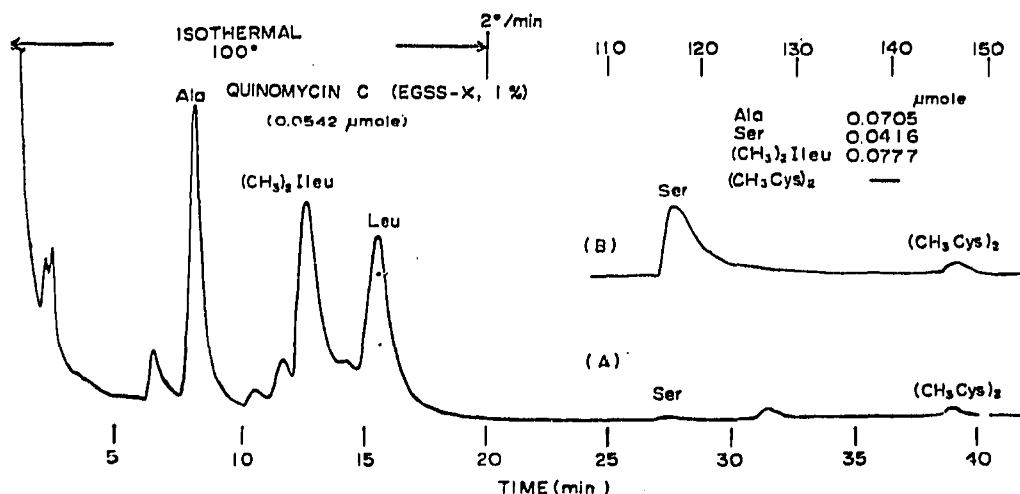

Fig. 4. Gas–liquid chromatography of a hydrolysate of 0.0542 µmole of Quinomycin C as N-trifluoroacetylamino acid methyl ester derivatives. $(CH_3)_2$Ileu = N,γ-dimethyl-alloisoleucine; $(CH_3Cys)_2$ = N,N′-dimethyl-cystine. (A) Pretreatment of the column was not carried out. (B) An excess amount of N-trifluoroacetyl-L-serine methyl ester was passed through the column at 140° before analysis. After isothermal analysis at 100° for 20 min, the temperature was raised to 210° at a rate of 2°/min. Other conditions are as described in the experimental section.

DISCUSSION

This discussion is concerned with the reaction of N-methylamino acid with ninhydrin, analysis by automatic amino acid analyzer and by gas–liquid chromatography.

Reaction of N-methylamino acid with ninhydrin

At the present time, the photometric ninhydrin method is the one most widely used of all the available colorimetric methods for the quantitative determination of α-amino acids. Although the photometric method can be expected to give reliable and reproducible results under a given set of conditions, there are some problems in the case of N-methylamino acids. The results obtained have shown that color yield of an N-methylamino acid with ninhydrin was poor compared to the parent amino acid (1.4–20 %), except for N-methyl-L-lysine (88 %) and methylamine (77 %). This may indicate, in general, that heating at 100° was not long enough for the reaction between both compounds to proceed to its maximum extent. The time of heating in the reaction-bath of the analyzer was designed to be 15 min when the flow rate of eluate was 30 ml/h. In a separate experiment on the time taken to complete the N-methylamino acid–ninhydrin reaction, it was shown that 20–30 min was necessary for the maximum color production at 100°. However, as far as these color yields were concerned, it is noteworthy that they were remarkably reproducible under the constant conditions used although the maximum deviations from averages were somewhat larger than in the case of the usual amino acids. The absorption spectrum of the developed color between an N-methylamino acid and ninhydrin was shown to be the same as that formed as a result of the usual amino acid–ninhydrin reaction[15]. This suggests that demethylation might occur before color development.

Analysis of N-methylamino acid by automatic amino acid analyzer

Because N-methylamino acids do not react completely with ninhydrin within 15 min at 100°, the determination of the N-methylamino acid by automatic amino acid analyzer was considered to be an unsuitable method. Results obtained, however, are better than those obtained by gas–liquid chromatography (compare Tables III and V). The reason for this might be the more rigid control of the conditions employed by the analyzer. N-Methyl-valine, N-methyl-alloisoleucine and N,γ-dimethyl-alloisoleucine were the N-methylamino acids which were the most difficult to determine by this method since the *C* constants of these compounds were the smallest among those analyzed (see Table II). If the reaction coil in the reaction bath of the automatic analyzer was changed for a long coil, in other words, if the eluent was heated at 100° for 20–30 min, analysis of N-methylamino acids by this method might be expected to be more accurate and complete.

Analysis of N-methylamino acids by gas–liquid chromatography

N-Trifluoroacetyl-N-methylamino acid methyl esters were chosen for this study since it was felt that these derivatives would have greatest volatility. Among the many different solid supports and stationary liquid phases tried for the separation of the trifluoroacetylamino acid methyl esters, Chromosorb W coated with 1 % w/w ethylene glycol succinate–siloxane copolymer (EGSS-X) proved most satisfactory.

Initial column temperature (100°), temperature program rate (2°/min), and nitrogen flow rate (20 ml/min) also had to be carefully adjusted to effect the best resolution of these compounds. The applicability of this method to N-methylamino acid analysis was demonstrated with a hydrolysate of the quinoxaline antibiotics (Table V) although some unsatisfactory results were found in the recoveries. However, the relative peak area for most of the amino acid derivatives was remarkably reproducible and was quite sufficient for the molar content in the sample compound to be determined (Fig. 4). By utilization of the peak area ratios, determination of the amino acid ratios in hydrolysates samples is possible. Determination of the usual amino acids in hydrolysates of peptides or proteins is now in progress in our laboratory by this method.

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SUMMARY

N^{α}-Monomethylamino acids were analyzed by an automatic amino acid analyzer and by gas–liquid chromatography. The positions of 17 N-methylamino acids on the chromatogram were indicated by the analyzer. Some quinoxaline antibiotics, such as Triostins and Quinomycins, which contained some N-methylamino acids as constituents, were used as samples for the determination. The analysis of N-methylamino acids by gas–liquid chromatography as N-trifluoroacetyl-N-methylamino acid methyl esters was also studied.

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