Journal of Chromatography, 140 (1977) 47–56

CHROM. 10,180

GAS CHROMATOGRAPHIC DETERMINATION OF METHYLGUANIDINE, GUANIDINE AND AGMATINE AS THEIR HEXAFLUOROACETYL-ACETONATES

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(Received February 3rd, 1977)

SUMMARY

Seven derivatives each of methylguanidine, guanidine and agmatine have been prepared, and the specificity and volatility of their gas chromatographic detection have been studied. The hexafluoroacetylacetonates have been found to be the most specific for the three guanidines, and are highly sensitive to alkali flame ionization and electron-capture detections. These derivatives are also fairly resistant to hydrolysis occurring in the derivatization process.

INTRODUCTION

Methylguanidine (MG), which has long been considered to occur widely in fresh beef and several fish in fairly high concentrations ranging from 60 to 1900 mg/kg (refs. 1–4), is known to be easily converted by nitrosation under acidic conditions into highly mutagenic and carcinogenic methylnitrosocyanamide and methylnitrosourea[5–7]. MG ingested in the diet may react with nitrite in the human stomach to form these potent carcinogens. Endo *et al.*[7] speculated that these substances may be possible etiologic factors in human gastric cancer. Concerning the occurrence of MG in foods, however, most of the studies were conducted in the 1930s[1–4], and the methods used for MG separation and analysis were overly complex, might have lacked specificity and might have generated MG. The lack of suitable analytical methods has hindered attempts to identify individual guanidine derivatives in various foods.

Recently, several methods have been reported for the analysis of various guanidino compounds in biological fluids, including ion-exchange[8–13], paper [14–17] and thin-layer chromatography[18], coupled with colorimetric determinations by either the Sakaguchi or Voges–Proskauer reactions, and gas–liquid chromatography

(GLC)[19–25] after the formation of volatile derivatives. The colorimetric determination, however, lacks the specificity needed to identify each guanidino compound, and also requires considerable sample manipulation and time. Some volatile derivatives subjected to GLC analysis also lack specificity or stability, and the derivative preparation seems rather complex.

In the present study, we have prepared several volatile derivatives of guanidines, *i.e.*, MG, guanidine (G) and agmatine (AG), and have compared their specificities of derivatization and volatilities as determined by GLC with alkali flame ionization detection (AFID). It was found that the hexafluoroacetylacetone (HFAA) derivatives were the most appropriate ones among the seven different types of derivatives tested so far. Methods for the preparation and identification of the HFAA derivatives of MG, G, and AG, and the conditions for the determination of these three derivatives by GLC, are described.

EXPERIMENTAL

Reagents

All of the reagents employed were commercial products of analytical grade and were used without further purification. Methylguanidine hydrochloride and agmatine sulphate were obtained from Sigma (St. Louis, Mo., U.S.A.), and guanidine hydrochloride was from Wako (Tokyo, Japan). Acetylacetone (AA), trifluoroacetylacetone (TFAA), hexafluoroacetylacetone (HFAA), acetic anhydride, trifluoroacetic anhydride and carbon disulphide were obtained from Wako, and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, specially purified grade) was from Pierce (Rockford, Ill., U.S.A.).

Derivative preparation

Acetyl derivatives of the three guanidines were prepared by use of acetic anhydride according to the method of Link *et al.*[26], and trifluoroacetyl derivatives using trifluoroacetic anhydride according to the method of Stalling and Gehrke[20]. AA derivatives were prepared according to Beyermann and Wisser[21], BSTFA (silyl) products according to Gehrke *et al.*[27] and isothiocyanates (isoCNS) by use of carbon disulphide according to Brandenberger and Hellbach[28]. TFAA derivatives were prepared as described for the HFAA derivatization using trifluoroacetylacetone instead of HFAA.

Preparation of HFAA derivarives

MG, G and AG were each prepared as solutions (1 mg/ml) in 50% ethanol. A 100-μl aliquot of a test solution was placed in a hard glass ampoule, and this was evaporated by blowing in nitrogen. Then, 50 μl each of pyridine and HFAA were added and the ampoule was heat-sealed, followed by heating at 120° for 1 h. After cooling the ampoule to room temperature, 1 ml of diethyl ether and 3 ml of 3 *N* HCl were added to the reaction mixture and this was shaken vigorously and centrifuged. A 5-μl aliquot of the ether layer was then injected into the GLC column.

Operating conditions for gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

A Shimadzu GC-5APF gas chromatograph, equipped with an AFID (KBr monocrystal-on-detector type[29]; GLC–AFID), and a Shimadzu GC-3BE, equipped

with a ^{63}Ni electron capture detector (GLC–ECD), were used. In order to compare the peak areas of various derivatives determined by GLC–AFID, a glass column (1 m × 3 mm I.D.) packed with 3% SE-30 on Chromosorb W (Applied Science Labs., State College, Pa., U.S.A.) was employed, and the column temperatures were varied from 70 to 200° depending on the derivatives examined. The mass spectra of the derivatives were determined by use of a Shimadzu-LKB 9000 gas chromatograph–mass spectrometer (GC–MS), equipped with a glass column (2 m × 3 mm I.D.) packed with 5% SE-30 on Chromosorb W AW DMCS.

The HFAA derivatives of the three guanidines were analyzed under the conditions shown in Table I.

TABLE I

OPERATING CONDITIONS FOR GLC AND GC–MS OF THE HFAA DERIVATIVES OF METHYLGUANIDINE, GUANIDINE AND AGMATINE

Operating conditions	*MG–HFAA and G–HFAA*	*AG–HFAA* [a]
GLC–AFID		
Apparatus	Shimadzu GC-5APF	
Detector	AFID (KBr monocrystal)	
Carrier gas	N_2	
Flow-rate (ml/min)		
N_2	40	
H_2	20	
air	700	
Temperature (°C)		
injection port	210	
detector	210	
column	120	170
Column	20% Versamid 900 on Chromosorb W (60–80 mesh), 1 m × 3 mm I.D., glass	3% SE-30 on Chromosorb W (60–80 mesh), 1 m × 3 mm I.D., glass
GLC–ECD		
Apparatus	Shimadzu GC-3BE	
Detector	^{63}Ni ECD	
Carrier gas	N_2	
Flow-rate (ml/min)	40	50
Temperature (°C)		
injection port	150	220
detector	100	160
column	100	160
Column	same as GLC–AFID	same as GLC–AFID
GC–MS		
Apparatus	Shimadzu–LKB, GC–MS 9000	
Detector	total ion collector	
Carrier gas	He	
flow-rate (ml/min)	30	
inlet pressure (kg/cm²)	3	
Temperature (°C)	100	220
separator	290	
ion source	290	
Electron current (eV)	20	70
Trap current (μA)	60	
Accelerating voltage (kV)	8	
Column	5% SE-30 on Chromosorb W AW DMCS (60–80 mesh), 2 m × 3 mm I.D., glass	

RESULTS AND DISCUSSION

Selection of the most desirable derivatives of the three guanidines for GLC analysis

Retention times, peak heights and peak shapes of seven derivatives of MG, G and AG were compared. The retention times obtained are shown in Table II. In addition, the peaks of all of the derivatives were confirmed by GC–MS. The HFAA derivatives were found to be the most suitable for the quantitative determination of the three guanidines by GLC since: (1) the derivatization with acetylacetone is highly specific for guanidines, according to the results of GC–MS analysis; (2) all of the three guanidines so far tested give derivatives with HFAA in high yield; (3) the HFAA derivatives are the most volatile of the three different acetylacetonates and (4) the HFAA derivatives exhibited the highest stability.

TABLE II

RETENTION TIMES OF VARIOUS VOLATILE DERIVATIVES OF METHYLGUANIDINE, GUANIDINE AND AGMATINE

Glass column (1 m × 3 mm I.D.) containing 3% SE-30 on Chromosorb W (60–80 mesh); carrier gas (nitrogen) flow-rate, 40 ml/min; detector, AFID.

Derivative	*Guanidine*	*Column temperature (°C)*	*Retention time (min)*
HFAA	MG	70	3.50
	G	70	3.50
	AG	170	4.15
TFAA	MG	90	2.70
	G	90	3.50
	AG	190	5.75
AA	MG	110	2.70
	G	110	2.95
	AG	70–300	—*
Trifluoroacetyl	MG	70	5.30
	G	70	5.40
	AG	190	1.90
Acetyl	MG	200	3.15
	G	200	4.30
	AG	70–300	—
BSTFA (silyl)	MG	140	3.70
	G	170	4.90
	AG	190	6.90
isoCNS	MG	70–300	—
	G	70–300	—
	AG	70–300	—

* No peak corresponding to the derivative was observed.

Derivatization conditions for MG, G and AG with HFAA

We initially prepared the HFAA derivatives of MG, G and AG according to the method of Erdtmansky and Goehl[24], which has been applied to the analysis of guanidino-type antihypertensive agents in the blood. However, it was found that the derivatization rates of the three guanidines were as low as 20%, and the rates were still *ca.* 50% even when the reaction mixtures were heated at 180° for 2 h. Erdtmansky and Goehl[24] used a reflux condenser during heating of the reaction mixture; when the

mixture was heated in a sealed vial instead of a condenser, it was found that the derivatization rates increased but were still insufficient. The reason for these low rates of derivatization was found to be hydrolysis of the products due to the presence of water in the reaction mixture. On the other hand, Beyermann and Wisser[21] added 100 mg of Na_2CO_3 and 1 ml of acetylacetone to the dry specimen in order to prepare the AA derivatives of MG and G, followed by heating under reflux at 140° for 30 min. We applied this method to the derivatization of the three guanidines with HFAA, and found that the HFAA derivatives were decomposed when more than 20 mg of Na_2CO_3 was added, and the yield of derivatives was markedly lowered. Mitchell[30] reported a method for the GLC analysis of Schiff bases of amino-acid methyl esters in which 1% pyridine in methanol was added as a catalyst. In the present study, the effect of addition of pyridine, $NaHCO_3$ and Na_2CO_3 on the HFAA derivatization was examined. As shown in Table III, pyridine gave the highest derivatization rates. Next, the effect of the volume of pyridine on the HFAA derivatization was examined, and the results shown in Fig. 1 indicate that the highest yield of HFAA derivatives could be obtained when 50 µl of pyridine was added to the reaction mixture.

For the extraction of HFAA derivatives from the reaction mixture, Erdtmansky and Goehl[24] employed benzene as the extractant. However, it was found that

TABLE III

EFFECT OF PYRIDINE, SODIUM BICARBONATE AND SODIUM CARBONATE ON THE DERIVATIZATION OF METHYLGUANIDINE, GUANIDINE AND AGMATINE WITH HFAA

Compound added	*Relative derivatization rate**		
	MG	*G*	*AG*
Control (no addition)	1.00	1.00	1.00
Pyridine	34.5	87.0	19.3
$NaHCO_3$	16.6	87.0	19.3
Na_2CO_3	19.6	40.8	13.7

* The derivatization rates of the controls were expressed as 1.00.

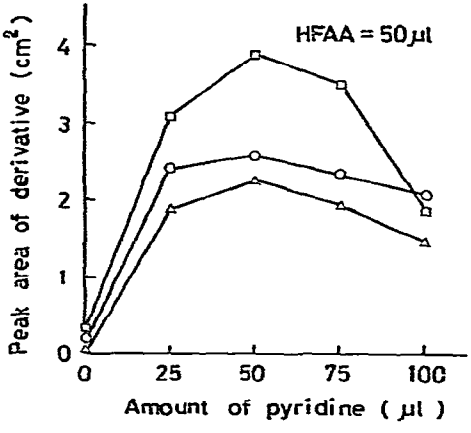


Fig. 1. Effect of the amount of pyridine on the derivatization of methylguanidine (○), guanidine (△) and agmatine (□) with HFAA. Heating condition, 120° for 60 min.

incomplete combustion of the benzene in the GLC detector chamber resulted in the production of a rather large amount of soot which apparently lowered the sensitivity of the AFID. The extraction rates of the HFAA derivatives with different solvents were thus examined. As can be seen from Table IV, diethyl ether gave the highest extraction rates among the 10 organic solvents tested.

TABLE IV

EXTRACTION RATES OF THE HFAA DERIVATIVES OF METHYLGUANIDINE, GUANIDINE AND AGMATINE IN 3 *N* HCl WITH DIFFERENT SOLVENTS

Solvent	*Relative extraction rate**		
	MG-HFAA	*G-HFAA*	*AG-HFAA*
Benzene	1.00	1.00	1.00
Toluene	0.89	0.93	—**
Cyclohexane	1.00	0.87	—
n-Hexane	0.97	0.89	—
n-Heptane	0.96	0.72	—
n-Pentane	1.23	1.09	—
Ethyl acetate	1.33	1.90	1.05
n-Butyl acetate	0.91	1.11	1.07
Diethyl ether	1.69	1.92	2.04
Methyl isobutyl ketone	1.03	1.14	—

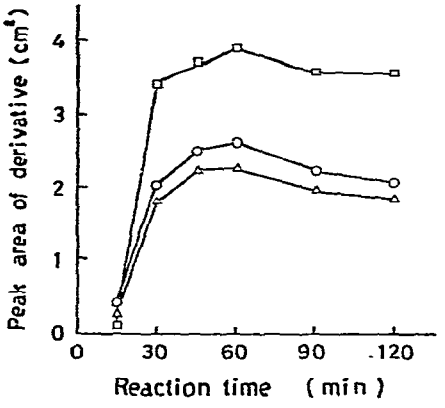
* The extraction rates with benzene were expressed as 1.00.
** Not examined.

However, not only the HFAA derivatives but also pyridine may be extracted from the reaction mixture by use of diethyl ether, and pyridine is also detected by the AFID. This must give rise to some error in the GLC–AFID determination of the HFAA derivatives. However, the acidity of the reaction mixture does not influence the extraction rates of the derivatives with diethyl ether. Thus, when 3 ml of 3 *N* HCl and 1 ml of diethyl ether were added to the reaction mixture, pyridine was completely retained in the aqueous layer.

We then examined the effects of heating temperature and time on the derivatization of the three guanidines with HFAA. As shown in Figs. 2 and 3, the highest derivatization rates were observed when the reaction mixtures were heated at 120° for 60 min.

Due to the difficulty in obtaining authentic HFAA derivatives of the three guanidines, the derivatization rates have thus far been expressed in terms of either the peak areas of the gas chromatograms (in Figs. 1, 2 and 3) or as relative derivatization rates (in Tables III and IV). Thus, the derivatization rates of the three guanidines were determined indirectly by estimating the remaining guanidines in the reaction mixtures, *viz.*, each 100 μg of MG, G or AG was derivatized with HFAA, and the remaining guanidine in the aqueous (lower) layer was determined colorimetrically using the modified Voges–Proskauer reaction according to Micklus and Stein[31]. Based on the remaining guanidines, the derivatization rates of MG, G and AG were found to be 100, 100 and 97.4%, respectively.

The mass spectra of the HFAA derivatives of the three guanidines are shown in Fig. 4; the probable structures of the compounds are also illustrated.



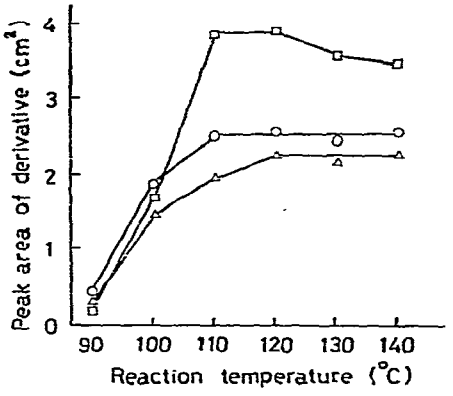


Fig. 2. Effect of the reaction time on the derivatization of methylguanidine (○), guanidine (△) and agmatine (□) with HFAA. Heating temperature, 120°.

Fig. 3. Effect of the reaction temperature on the derivatization of methylguanidine (○), guanidine (△) and agmatine (□) with HFAA. Heating time, 60 min.

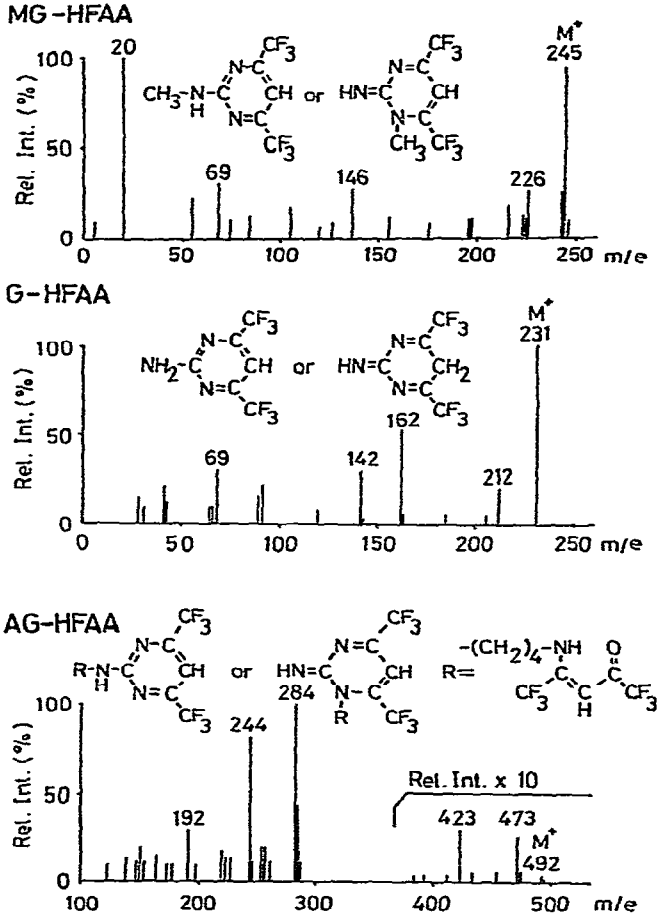


Fig. 4. Mass spectra of the HFAA derivatives of methylguanidine, guanidine and agmatine, and their probable structures.

GC determination of the HFAA derivatives

For the detection of nitrogenous substances, it is known that the AFID is much more sensitive than the hydrogen flame ionization detector (FID). It is also known that halogenated compounds such as HFAA derivatives are quite sensitive to the ECD as compared with the AFID. In the present study, however, most of the experiments were conducted with GLC–AFID because the derivatives of the three guanidines tested included compounds other than halogenated ones, and, moreover, the sensitivity of GLC–AFID to the test compounds was found to be almost the same as that of GC–MS.

The conditions for GC determination of the HFAA derivatives of guanidines were examined. The retention times obtained with different column packings are shown in Table V. It was found that all of the HFAA derivatives of the three guanidines could not be analyzed under the same column conditions because the molecular weight of the HFAA derivative of AG (AG–HFAA) is much larger than those of MG–HFAA and G–HFAA, and, in addition, the volatility of AG–HFAA is fairly low compared to the other two derivatives. Consequently, we selected a column containing 20% Versamid 900 for the analysis of MG–HFAA and G–HFAA, and for AG–HFAA we used a column of 3% SE-30. The gas chromatograms determined by AFID of the three guanidine HFAA derivatives are shown in Fig. 5.

TABLE V

RETENTION TIMES OF THE HFAA DERIVATIVES OF METHYLGUANIDINE, GUANIDINE AND AGMATINE OBTAINED WITH THE DIFFERENT TYPES OF COLUMN PACKINGS

Glass columns (1 m × 3 mm I.D.) were used.

Column		*Retention time (min)*		
Packing	*Temperature (°C)*	*MG–HFAA*	*G–HFAA*	*AG–HFAA*
3% SE-30	70 (170)*	3.50	3.50	4.15
3% OV-17	70 (170)*	4.35	4.45**	3.70
20% Versamid 900	120	2.65	4.55	—
25% PEG-6000	120	8.15	2.05	—
5% Diethyleneglycol adipate	120	2.50	0.95	—

* The column temperature for AG–HFAA analysis.

** A tailing peak was observed.

Fig. 6 shows the calibration curves for the HFAA derivatives of the three guanidines; these indicate linear relations between the peak areas and amounts of the respective derivatives over ranges of 5–40 ng for MG and G and 15–50 ng for AG. The minimum detection limits when using GLC–AFID were 5 ng for MG and G and 15 ng for AG, and when using GLC–ECD were 50 pg for MG and G and 150 pg for AG.

The proposed HFAA derivatization method may be applicable to the estimation of these guanidino compounds at levels of parts per billion (μg/kg) in foods. The conditions for the analysis of these compounds in foods are currently being investigated in further detail.

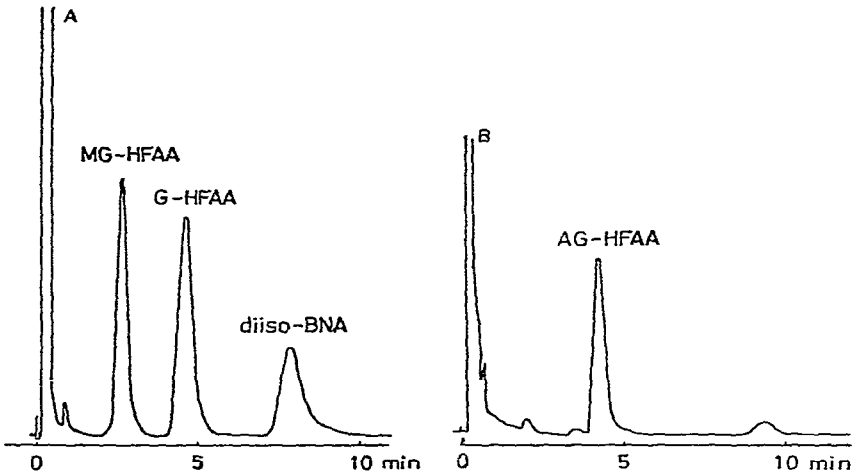


Fig. 5. Gas chromatograms of the HFAA derivatives of methylguanidine and guanidine (A) and agmatine (B). (A) Column packing, 20% Versamid 900; column temperature, 120°. (B) Column packing, 3% SE-30; column temperature, 170°. Diisobutylnitrosamine (diiso-BNA) was used as the internal standard.

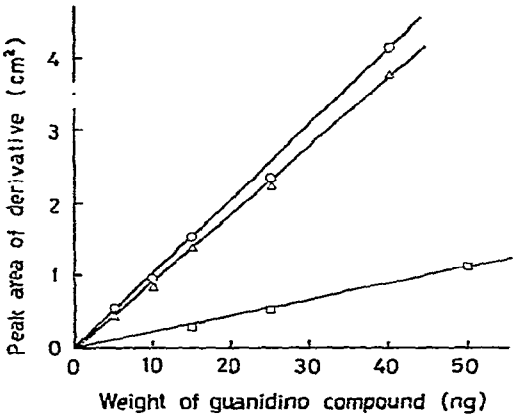


Fig. 6. Calibration curves for the HFAA derivatives of MG (○), G (△) and AG (□).

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan, 1975.

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