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GAS CHROMATOGRAPHIC DETERMINATION OF MICRO-AMOUNTS OF CYCLAMATES

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SUMMARY

It was found that triethylammonium cyclamate is converted into N-heptafluorobutyrylcyclohexylamine in a high and constant yield by reaction with heptafluorobutyric anhydride at 90° for 1 h, and gas chromatography of the product gives a sharp peak that is highly sensitive to an electron capture detector. A useful method for the micro-determination of cyclamates was established by combining this reaction with gas chromatography.

INTRODUCTION

A number of methods have been reported for the determination of cyclamic acid and cyclamates in foods and drugs[1]. Of these methods, the gas chromatographic analysis of cyclamates, which has various defects, has been used on the products formed by acid hydrolysis[2] or nitrous acid degradation[3–5] of cyclamates.

As cyclamic acid (cyclohexanesulphamic acid) is a sulphuric acid amide of cyclohexylamine, it would be expected to form a carboxylic acid cyclohexylamide directly by transamidation with a carboxylic acid. We have found from our study on the transamidation that triethylammonium cyclamate is converted into N-heptafluorobutyrylcyclohexylamine with an almost quantitative yield by reaction with heptafluorobutyric anhydride for 1 h at 90°.

This paper reports the formation of N-heptafluorobutyrylcyclohexylamine from cyclamic acid and the gas chromatographic analysis of cyclamates in micro-amounts.

EXPERIMENTAL

Reagents and solutions

Cyclamates. Commercial sodium cyclamate was purified by recrystallization from ethanol–water. The purified sodium cyclamate melts at 280° with decomposition. Crystalline cyclamic acid, m.p. 169–170°, was prepared by converting

sodium cyclamate into its free form with a cation exchanger, followed by recrystallization from water.

Heptafluorobutyric anhydride. Commercial heptafluorobutyric anhydride was re-distilled over phosphorus pentoxide and the fraction of b.p. 106–112° was used[6].

N-Heptafluorobutyrylcyclohexylamine. A solution of cyclohexylamine (0.5 ml) in dichloromethane (2 ml) was mixed with heptafluorobutyric anhydride (1.5 ml) dissolved in dichloromethane (2 ml) and allowed to react for 1 h at room temperature. The reaction mixture was evaporated under reduced pressure and the solid residue was recrystallized from ethanol–water, giving 0.76 g of colourless needles, m.p. 77–79°. The elemental analyses for C, H and N corresponded to the formula $C_{10}H_{12}NOF_7$.

N-Trifluoroacetanilide. N-Trifluoroacetanilide (m.p. 85–86°) was prepared by N-trifluoroacetylation of aniline, followed by recrystallization from ethanol–water[7].

Internal standard solutions. Solutions of diphenyl (0.015, 0.03, 0.2, and 0.5%) in ethanol and N-trifluoroacetanilide (0.045%) in ethanol were prepared (see Table I).

Standard solutions of N-heptafluorobutyrylcyclohexylamine. Solutions containing amounts of N-heptafluorobutyrylcyclohexylamine in the ranges shown in Table I were prepared by using the corresponding internal standard solution.

TABLE I

STANDARD SOLUTIONS OF N-HEPTAFLUOROBUTYRYLCYCLOHEXYLAMINE

Concentration of N-heptafluorobutyrylcyclohexylamine (mg/ml)	*Concentration of internal standard in ethanol (%)*		*Detector*
	Diphenyl	*N-Trifluoro-acetanilide*	
1.5 –6	0.5	—	FID
0.4 –1.5	0.2	—	FID
0.1 –0.4	0.03	—	FID
0.02 –0.1	0.015	—	FID
0.005–0.02	—	0.045	ECD

Analytical methods

Thin-layer chromatography (TLC). A glass plate (10 × 10 cm) coated with a 0.25-mm layer of Kieselgel G (Merck, Darmstadt, G.F.R.) suspended in a 1.5% aqueous solution of soluble starch was dried in air, then heated at 60° for 1 h. Samples were spotted on the plate, which was developed by the ascending technique using one of the following solvents: (a) acetone–benzene (3:7); (b) acetone–*n*-hexane–benzene (1:2:5); and (c) toluene–ethyl acetate (3:1). After drying in air, the developed plate was heated at 100° for 15 min, kept in a closed tank containing two or three drops of *tert.*-butyl hypochlorite solution (10% in acetic acid) at room temperature for 10 min, then sprayed with 1% potassium iodide solution in order to render visible the spot of N-heptafluorobutyrylcyclohexylamine. TLC analysis of cyclamates was carried out on a plate coated with Avicel SF, as previously reported[8].

Gas chromatography (GC). GC analysis was carried out on a Shimadzu GC-4BM gas chromatograph equipped with a flame ionization detector (FID) or an elec-

tron capture detector (ECD), employing a 2 m × 3 mm I.D. glass column packed with 2% silicone XF-1105 on 60–80 mesh Gas-Chrom P.

Gas chromatography–mass spectrometry (GC–MS). GC–MS was carried out with a JMS-D-100 mass spectrometer equipped with a JEOL JGC-20K gas chromatograph employing a 1 m × 3 mm I.D. glass column packed with 3% OV-1 on 80–100 mesh Chromosorb W AW.

Examination of the reaction of cyclamates with heptafluorobutyric anhydride

Cyclamates (free acid or salt form, 5 mg) and heptafluorobutyric anhydride (1 ml) were introduced into a 12 × 1 cm I.D. test-tube, which was sealed. After heating at 90° for 1 h, the tube was cooled and opened. The contents were evaporated by passing nitrogen gas through a capillary under gentle warming, then dried over sodium hydroxide pellets *in vacuo* for 30 min. The residue was mixed with 1.5 ml of a 0.5% solution of diphenyl in ethanol and centrifuged at 550 *g* for 5 min. The supernatant was submitted to GC or GC–MS.

RESULTS AND DISCUSSION

Characterization of products formed by the reaction of cyclamates with heptafluorobutyric anhydride

It was shown by both TLC and GC analyses that the reaction of triethylammonium cyclamate with heptafluorobutyric anhydride gives N-heptafluorobutyrylcyclohexylamine as the sole product in nearly quantitative yield. R_F values of the product agreed with those of authentic N-heptafluorobutyrylcyclohexylamine [0.63 with solvent (a), 0.71 with solvent (b) and 0.81 with solvent (c)]. A gas chromatogram of the product is given in Fig. 1, and is identical with that of authentic

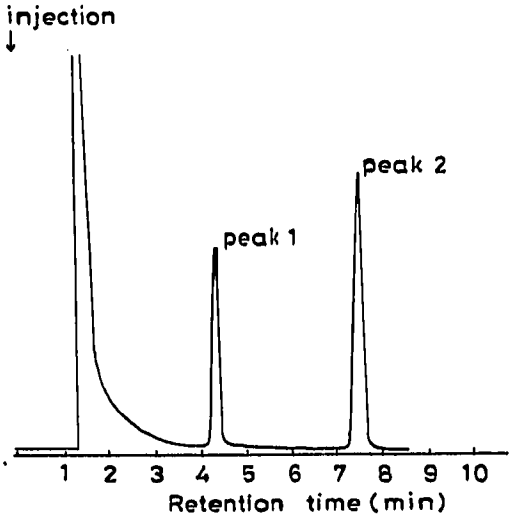

Fig. 1. Gas chromatogram of N-heptafluorobutyrylcyclohexylamine. Operating conditions: column temperature, 115°; injector, 170°; detector (FID), 170°; nitrogen flow-rate, 40 ml/min. Peak 1 = N-heptafluorobutyrylcyclohexylamine; peak 2 = diphenyl (internal standard).

TABLE II

EFFECT OF TEMPERATURE ON THE REACTION OF TRIETHYLAMMONIUM CYCLAMATE WITH HEPTAFLUOROBUTYRIC ANHYDRIDE

Reaction time: 60 min.

No. of experiment	*Formation of N-heptafluorobutyrylcyclohexylamine (%)*		
	80°	*90°*	*100°*
1	80.25	83.87	82.64
2	74.79	83.12	84.69
3	75.13	84.01	84.01
4	68.64	83.52	80.25
5	67.61	83.63	80.59
Mean	73.29	83.63	82.44
Standard deviation	4.504	0.344	1.777

N-heptafluorobutyrylcyclohexylamine. Further, GC–MS of the single peak revealed the presence of a molecular ion peak (M^+) at *m/e* 295 together with peaks[9] at *m/e* 55, 82, 214 and 252.

Examination of optimum reaction conditions for formation of N-heptafluorobutyrylcyclohexylamine

Triethylammonium cyclamate was separately reacted at 80°, 90° and 100° for 60 min. The degree of formation of N-heptafluorobutyrylcyclohexylamine was determined by GC analysis using a calibration graph prepared with the standard N-heptafluorobutyrylcyclohexylamine solutions. As can be seen from Table II, reaction at 90° gave the best result.

In order to establish the most suitable form of cyclamates, free cyclamic acid, its sodium salt and its triethylammonium salt were reacted separately at 90°, and the time courses of their reactions were examined. As shown in Fig. 2, the highest and

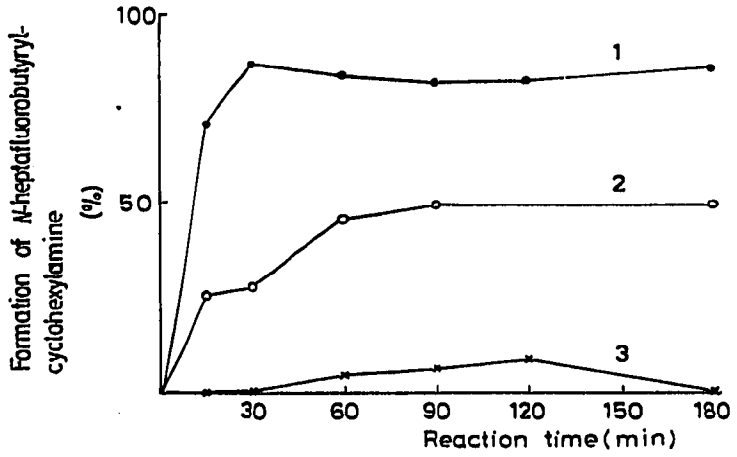

Fig. 2. Time courses of the formation of N-heptafluorobutyrylcyclohexylamine from cyclamates. 1 = Triethylammonium cyclamate; 2 = sodium cyclamate; 3 = free cyclamic acid.

most constant yield of N-heptafluorobutyrylcyclohexylamine was given when triethylammonium cyclamate was used. A reaction time of 60 min was selected because of its practical convenience.

A number of methods for the clean-up of cyclamates in samples have been described, and most of them require a final extraction of free cyclamic acid with organic solvents after several purification steps. Therefore, on application of the present reaction, free cyclamic acid in solution, which was obtained by extracting samples, was neutralized with triethylamine, then evaporated, and the residue submitted to successive reaction with heptafluorobutyric anhydride.

Determination of cyclamate

Preparation of calibration graphs. Calibration graphs were prepared for the five ranges of N-heptafluorobutyrylcyclohexylamine shown in Table I. Good linearity was obtained in all instances, and two of them, in which an ECD and FID, respectively, were used as the detector, are shown in Figs. 3a and 3b.

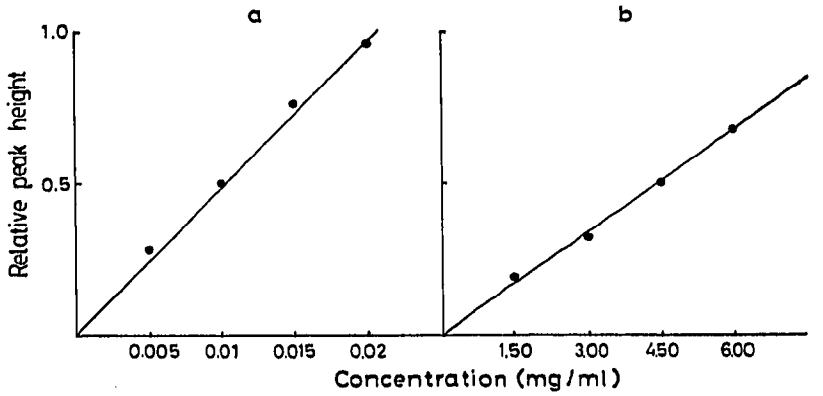

Fig. 3. Calibration graphs for N-heptafluorobutyrylcyclohexylamine in the concentration ranges (a) 0.005–0.02 mg/ml using an ECD and (b) 1.50–6.00 mg/ml using an FID.

Analytical procedure. A constant volume (containing 0.006–7 mg of free cyclamic acid) of the free cyclamic acid solution, which was extracted from the samples by an appropriate clean-up method, is introduced into a test-tube (12 × 1 cm I.D.) and neutralized with triethylamine, then evaporated to dryness by passing nitrogen gas through a capillary. The reaction and subsequent operations are carried out as described under *Examination of the reaction of cyclamates with heptafluorobutyric anhydride.* The residue obtained is dissolved in 1.5 ml of the corresponding internal standard solution. After centrifugation, an aliquot (0.6 μl for the FID and 10 μl for the ECD) of the supernatant is submitted to GC .

Calculation. The amount of N-heptafluorobutyrylcyclohexylamine obtained from a calibration graph is converted into the amount of free cyclamic acid according to the equation

$$A \cdot \frac{M_C}{M_H} \cdot \frac{100}{83.63} \cdot 1.5 = B$$

where

A = amount of N-heptafluorobutyrylcyclohexylamine;
B = amount of free cyclamic acid;
M_C = molecular weight of free cyclamic acid;
M_H = molecular weight of N-heptafluorobutyrylcyclohexylamine.

The numeral 83.63 is the average percentage formation of N-heptafluorobutyrylcyclohexylamine from cyclamic acid (see Table II).

CONCLUSION

The use of cyclamates as food additives has been forbidden in many countries since the autumn of 1969 owing to their possible carcinogenic properties. There have been many estimations of the carcinogenicity of cyclamates, and intensive metabolic and carcinogenic studies with experimental animals have been carried out.

A number of methods for the determination of cyclamates contained in foods and drugs in measurable amounts have been reported but, as far as we are aware, no method suitable for the determination of cyclamates in samples available in only a limited amount, such as body fluids, organs and excreta of animals, has been reported. It seems that the method described here is more useful than present methods for the micro-determination of cyclamates.

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