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GAS CHROMATOGRAPHY OF URINARY KYNURENINE

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SUMMARY

Urinary kynurenine was converted by heating it with barium hydroxide into 2-aminoacetophenone, which was analyzed as its trifluoroacetyl derivative by gas chromatography. The peak that had the same retention time as that of authentic 2-trifluoroacetylaminoacetophenone was investigated by combined gas chromatography–mass spectrometry.

INTRODUCTION

It has been reported that an increase in the amount of the urinary tryptophan metabolites is associated with some diseases. In previous papers[1,2], a procedure was developed for the gas chromatographic (GC) determination of urinary anthranilic acid. In the present paper, two methods for the GC determination of urinary kynurenine are described, one being a simple procedure and the other a specific procedure but more complicated.

EXPERIMENTAL

Apparatus and conditions

The gas chromatograph was the same as that described in a previous paper[2] and the conditions for separation are shown later in Fig. 2.

Gas chromatography–mass spectrometry (GC–MS)

The mass spectra of 2-trifluoroacetylaminoacetophenone and the experimental peaks obtained from kynurenine and urine samples were measured on a Hitachi Model RMU-6E mass spectrometer. The operating conditions were: ionizing potential, 70 eV; ion accelerating potential, 1.8 kV; ion source temperature, 220°; total emission, 80 μA; target current, 70 μA; and multiplier potential, 3 kV. All samples were introduced into the ionization chamber through a Hitachi Model K-53 gas chromatograph. A 2.0-m glass column packed with 7% XF-1105 on Gas-Chrom Z (80–100 mesh) was used, and the temperature was maintained at 140°.

Standard procedure

Method A. In this simple and rapid method, 10 ml of saturated barium hydroxide and 1 g of barium hydroxide powder were added to 10 ml of urine sample, and the mixture was heated for 20 min on a boiling water-bath under reflux. After cooling in an ice-bath, 1.0 ml of chloroform solution containing a known amount of *p*-xylene dichloride (an internal standard) was added and the 2-aminoacetophenone produced was extracted with two 5-ml portions of chloroform. The combined chloroform extracts were evaporated to dryness at room temperature under reduced pressure, and the residue was treated with 1 drop of ethyl acetate and 2 drops of trifluoroacetic anhydride. After 10 min, the reaction mixture was injected into the gas chromatograph.

Method B. In this specific but more complicated method, 0.5 ml of concentrated hydrochloric acid was added to 10 ml of urine sample and the mixture was heated for 1 h on a water-bath at 80° under reflux so as to hydrolyze the conjugated 2-aminoacetophenone. Some acidic components were removed by extraction with 10 ml of chloroform, then the aqueous solution was made alkaline with 1 ml of 5 *N* sodium hydroxide, 10 ml of saturated barium hydroxide and some basic components including 2-aminoacetophenone produced by hydrolysis were removed by extraction with 10 ml of chloroform. Then, 1 g of barium hydroxide powder was added and the mixture was treated according to *Method A.*

Preparation of the trifluoroacetyl derivative of 2-aminoacetophenone

Excess of trifluoroacetic anhydride was added to 300 mg of pure 2-aminoacetophenone in ethyl acetate and the mixture was allowed to stand overnight at room temperature. A small volume of water was added to the solution and the precipitate produced was filtered off. The melting point of the product was 108°. Calculated for $C_{10}H_8O_2NF_3$: C, 51.96; H, 3.49; N, 6.059%. Found: C, 51.98; H, 3.63; N, 6.31%.

RESULTS AND DISCUSSION

It has been reported that kynurenine is converted by heating in alkaline solution into 2-aminoacetophenone[3]. However, there are no reports on the quantitative conditions for the conversion of kynurenine into 2-aminoacetophenone and the GC analysis of urinary kynurenine. The optimum conversion conditions given in *Method A* were established from preliminary experiments. A urine sample was collected in a bottle and acidified to 0.5 *N* with hydrochloric acid, which was stable for at least 48 h. For the decompositon of kynurenine, barium hydroxide was the most suitable of several basic reagents studied, such as sodium hydroxide, saturated barium hydroxide, disodium phosphate and their mixtures. The conversion ratio of kynurenine to aminoacetophenone increased with increase in the barium hydroxide concentration in the reaction solution, and became constant by adding 10 ml of saturated barium hydroxide and more than 0.5 g of barium hydroxide powder to 10 ml of urine sample, as shown in Fig. 1.

Next, the effects of temperature and heating time on the conversion of kynurenine were examined. In an ice-bath, kynurenine was stable even in strongly alkaline solution, while in a boiling water-bath, kynurenine was readily converted into 2-aminoacetophenone and the conversion ratio was constant during heating times

between 10 and 40 min. The 2-aminoacetophenone produced was readily extracted with chloroform in a basic medium, and loss of 2-aminoacetophenone and *p*-xylene dichloride, which was added later as an internal standard, was negligible in the step of evaporation to dryness at room temperature at reduced pressure. From these results, *Method A* was established as described above. The recovery obtained when using *Method A* was calculated to be 98.9±1.4% (S.D.) for five 10-ml portions of identical sample solutions containing 30 μg of kynurenine per 10 ml, as shown in Table I.

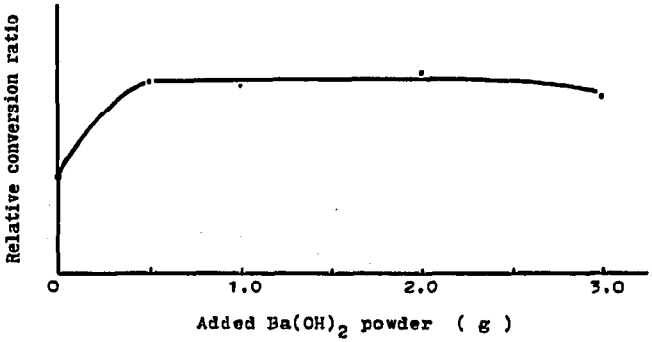


Fig. 1. Effect of barium hydroxide concentration on the conversion of urinary kynurenine into 2-aminoacetophenone. To 10 ml of urine sample, 10 ml of saturated barium hydroxide and various amounts of barium hydroxide powder were added and the mixture was treated according to *Method A*.

TABLE I

RECOVERY OF KYNURENINE BY *Method A*

Sample	*Taken (μg)*	*Found (%)*
1	30	97.0
2	30	99.3
3	30	100.0
4	30	100.3
5	30	98.0
Average		98.9

A gas chromatogram obtained from 10 ml of 0.1 *N* hydrochloric acid solution containing 20 μg of kynurenine with *Method A* is illustrated in Fig. 2. The peak X in Fig. 2 has the same retention time as that of the trifluoroacetyl derivative of authentic 2-aminoacetophenone, which suggests that the product formed by heating kynurenine with barium hydroxyde is 2-aminoacetophenone.

The GC–MS spectral data of peak X are shown in Fig. 3 and were also identical with those of the authentic trifluoroacetyl derivative of 2-aminoacetophenone.

The ion at *m/e* 231 indicates the molecular weight and the peak at *m/e* 216 corresponds to the loss of CH_3 and the peak at *m/e* 162 corresponds to the loss of CF_3 from the side-chain, as illustrated in Fig. 3. From the above results and elementary

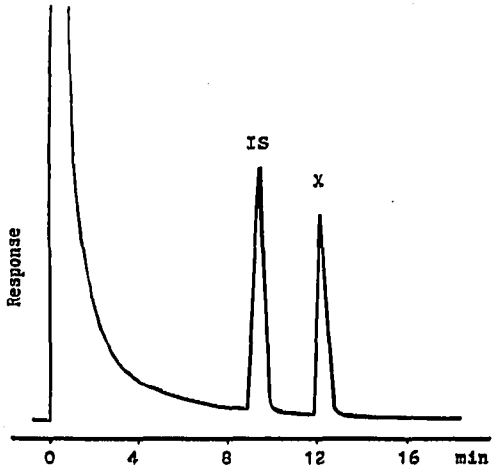


Fig. 2. Separation of (IS) *p*-xylene dichloride (internal standard) and (X) 2-trifluoroacetylaminoacetophenone (derived from kynurenine by *Method A*). Gas chromatograph: Shimadzu, Model GC-4APF, equipped with a hydrogen flame ionization detector. Column: 7% GE-XF 1105, 3.0 m × 3 mm I.D. stainless steel, 140°; sample size: 1.0 μl; chart speed: 0.5 cm/min.

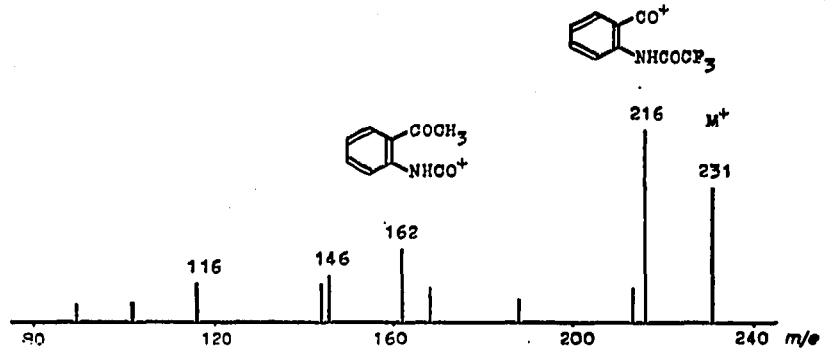
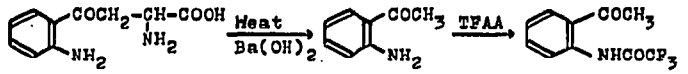


Fig. 3. GC–MS spectra of peak X in Fig. 2.

analysis, the peak X is concluded to be 2-trifluoroacetylaminoacetophenone (TFAA) (Scheme 1).



p-Xylene dichloride was suitable as an internal standard and the calibration curve was linear and passed through the origin in the range 10–50 μg of kynurenine. *Method A* was applied to the urine samples and a gas chromatogram obtained from 10 ml of urine is illustrated in Fig. 4.

The retention time and the GC–MS pattern of peak Y in Fig. 4 agreed completely with those of authentic 2-trifluoroacetylaminoacetophenone, which shows that peak Y represents a single component and corresponds to 2-aminoacetophenone derived from urinary kynurenine. *Method A* is very simple, but has the disadvantage that co-existing 2-aminoacetophenone interferes in the determination of kynurenine.

Recently, Kaseda *et al.*[4] reported that administration of tryptophan to hens or rats resulted in the occurrence of urinary 2-aminoacetophenone. However, neither the free form nor conjugated forms of 2-aminoacetophenone have been detected in normal urines. *Method A* is therefore useful enough for the determination of urinary kynure-

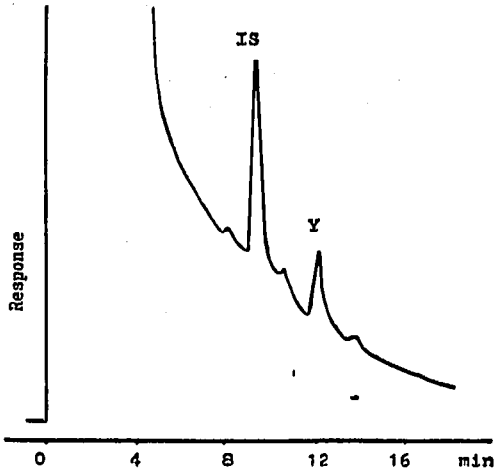


Fig. 4. GC of urine sample by *Method A*. Conditions as in Fig. 2.

TABLE II

RECOVERIES FROM URINE SAMPLES SPIKED WITH KYNURENINE

Sample	*Method A*		*Method B*	
	Taken (μg)	*Found (%)*	*Taken (μg)*	*Found (%)*
1	20.00	77.85	20.00	78.36
2	20.00	77.50	20.00	79.57
3	20.00	76.65	20.00	79.95
4	20.00	77.60	20.00	76.68
5	20.00	78.60	20.00	78.92
Average		77.64		78.70
σ(%)		0.90		1.63

nine. When the removal of a disturbance by co-existing urinary 2-aminoacetophenone is required, the free form can be removed by prior extraction with chloroform in cold alkaline solution by *Method A*, in which kynurenine is proved not to be decomposed. In addition, *Method B* was effective for the removal of the conjugated 2-amino-acetophenones as described above. The 2-aminoacetophenone-N-acetate, which seems to be the most stable of possible conjugates, was hydrolyzed by heating for 1 h at 80° in 0.5 *N* HCl. Hence 2-aminoacetophenone conjugates, if any exist, are completely removed by *Method B*. *Method B* is complicated, but specific. The recoveries were determined by *Methods A* and *B* on five 10-ml portions of an identical urine sample spiked with kynurenine (2 μg/ml), and the values obtained are shown in Table II.

Different urine samples resulted somewhat in variation among their recovery values, so that it is desirable to utilize the working curve prepared by using the experimental urine. These techniques were applied to the determination of kynurenine in some normal human urines and the results are given in Table III.

TABLE III

DETERMINATION OF FREE 2-AMINOACETOPHENONE, CONJUGATED 2-AMINOACETOPHENONE AND KYNURENINE IN SOME NORMAL HUMAN URINES

Sex	*Age*	*Method A*		*Method B*	
		2-AAP (free)	*Kynurenine*	*2-AAP (conjugated)*	*Kynurenine*
M	43	—	3.38	—	4.80
F	25	—	3.38	—	6.00
M	23	—	5.56	—	5.33
M	23	—	5.14	—	5.01

The results in Table III show that kynurenine is excreted in the range of 3.0–6.0 μg per 10 ml of urine sample and no 2-aminoacetophenone, free or conjugated, was found to be present in normal urines. It is not evident why a considerable discrepancy was observed between the values obtained by *Methods A* and *B* in the case of sample No. 2; it would probably be of little significance. Investigations are currently being performed in our laboratory on an application of this technique to the GC analysis of urinary 3-hydroxykynurenine.

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