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GAS CHROMATOGRAPHY OF URINARY ANTHRANILAMIDE

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SUMMARY

A procedure for the gas chromatographic determination of urinary anthranilamide was developed. Anthranilamide extracted with chloroform from an aqueous sample solution was separated gas chromatographically as the trifluoroacetyl derivative, which was concluded to be 2-trifluoromethyl-3,4-dihydro-4-quinazolone from gas chromatographic–mass spectrometric data. This technique was successfully applied to the urine of a rat to which anthranilic acid had been administered.

INTRODUCTION

It has been reported that the intraperitoneal administration of anthranilic acid to rats results in the excretion of urinary anthranilamide[1], and anthranilamide was isolated from urine and identified by paper chromatography, IR spectra, various colour reactions and elemental analysis. In the present paper, the gas chromatography (GC) of urinary anthranilamide was investigated as one of a series of GC investigations[2–5] of tryptophan metabolites.

EXPERIMENTAL

Apparatus and conditions

A Shimadzu Model GC-3BF gas chromatograph equipped with a hydrogen flame ionisation detector (HFID) was used, and the conditions for separation are shown later in Fig. 1. A Shimadzu Model GC-4APE gas chromatograph equipped with an electron capture detector (ECD) was also used, and the conditions are described later in Fig. 4.

Gas chromatography–mass spectrometry (GC–MS)

The mass spectra of trifluoroacetylated anthranilamide, ethyl anthranilate and the experimental peaks obtained from rat urine samples were measured on a Hitachi Model RMU-6E mass spectrometer. The operating conditions are the same as those in the previous paper[5].

Standard procedure

A 5-ml volume of 60% sodium hydroxide solution and 3.0 g of sodium chloride were added to 10 ml of sample solution acidified with hydrochloric acid to about pH 1, and the mixture was extracted with 10 ml of chloroform containing a known amount of *p*-xylene dichloride as an internal standard by shaking for 5 min with a mechanical shaker. The chloroform layer was transferred to a centrifuge tube and centrifuged for 5 min at 2000 r.p.m., after which the chloroform layer was removed with the aid of a syringe equipped with a long needle, transferred to a 10-ml pear-shaped flask and evaporated just to dryness at room temperature and reduced pressure. The residue was immediately dissolved in 1 drop of freshly prepared dioxane–pyridine mixture (6:1) and then trifluoroacetylated at room temperature with 1–2 drops of trifluoroacetic anhydride. A 1-μl volume of the solution was injected on to the gas chromatograph.

RESULTS AND DISCUSSION

Of the several solvents tested for the extraction of anthranilamide from aqueous solution, chloroform proved to be the most suitable, as it has a moderate vapour pressure for extraction and is clearly separated from the aqueous layer. When saturated with 3 g of sodium chloride, the extraction of anthranilamide with 10 ml of chloroform from 15 ml of sample solution in 20% sodium hydroxide solution was found to be 92%.

Next, effective conditions for the trifluoroacetylation of anthranilamide were examined. A small amount of anthranilamide was dissolved in 1 drop of various solvents, such as ethyl acetate, chloroform, dimethylformamide, tetrahydrofuran, ether, hexane, acetonitrile, dioxane and acetone, followed by treatment with 2 drops of trifluoroacetic anhydride at room temperature. The resulting mixture gave two peaks on the gas chromatogram, at 6 and 9.5 min (Fig. 1a). When trifluoroacetylated in dioxane containing a small amount of basic reagent, a single peak with a retention

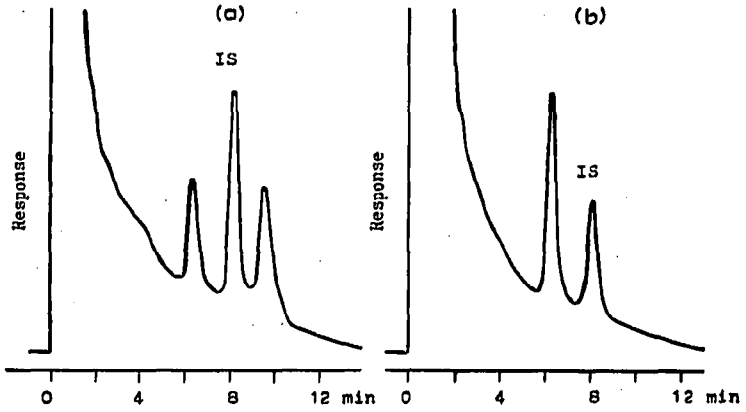


Fig. 1. Trifluoroacetylation of anthranilamide: (a) treated with 1 drop of acetone and 2 drops of trifluoroacetic anhydride; (b) treated with 1 drop of dioxane–pyridine mixture (6:1) instead of acetone. IS (internal standard), *p*-xylene dichloride. Conditions: 10% GE-XF 1105 on Gas-Chrom Z (80–100 mesh), 1.5 m × 3 mm I.D. stainless-steel column, 130°, HFID.

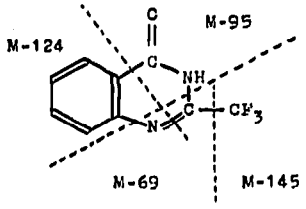
time of 6 min was found (Fig. 1b). One drop of freshly prepared dioxane–pyridine mixture (6:1) was suitable as a basic catalyst. The use of too much pyridine resulted in a precipitate, which disturbed injection of the reaction solution into the gas chromatograph, while too little pyridine failed to form the derivative quantitatively. The use of triethylamine as a basic reagent was unsuitable because it led to a broad solvent peak, which interfered with a peak of the derivative. *p*-Xylene dichloride was suitable as an internal standard, and its solution in chloroform was stable for at least 12 days in the cool. The calibration curve was linear and passed through the origin in the range 10–40 μg of anthranilamide. Recoveries were determined on five 10-ml portions of an identical urine sample spiked with anthranilamide (4 μg/ml) and the values obtained were calculated to be 101.1 ±4.1% (S.D.), as shown in Table I.

The GC–MS spectrum of trifluoroacetylated anthranilamide is given in Fig. 2. A molecular ion was observed at *m/e* 214 and the peak at *m/e* 145 corresponds to the

TABLE I

RECOVERY OF ANTHRANILAMIDE FROM SPIKED CONTROL URINE

Sample	*Taken (μg)*	*Found (%)*
1	40.0	104.2
2	40.0	99.2
3	40.0	94.7
4	40.0	104.3
5	40.0	103.3
Average		101.1



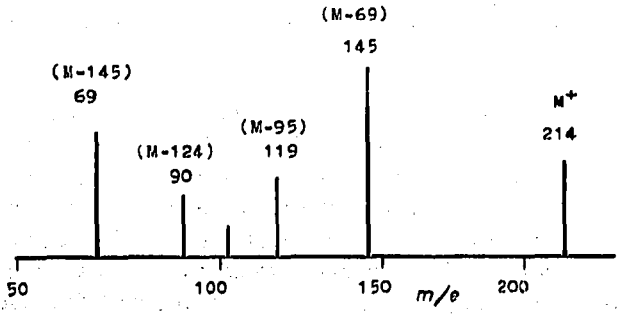
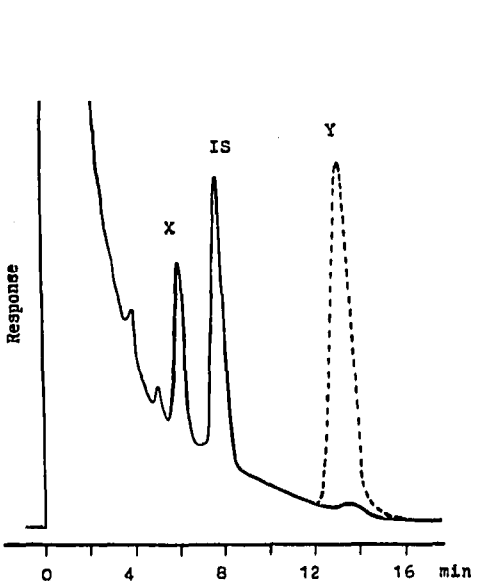


Fig. 2. GC–MS spectrum of the trifluoroacetyl derivative of anthranilamide.

loss of CF_3. Other prominent fragments are reasonably illustrated in Fig. 2. From these results, the derivative formed was concluded to be 2-trifluoromethyl-3,4-dihydro-4-quinazolone.

This procedure was applied to the urine of a rat to which anthranilic acid had been administered. Anthranilic acid (100 mg) was administered intraperitoneally to a rat and the urine was collected in 10 ml of 1 *N* hydrochloric acid over 24 h (a total of 24.5 ml was obtained). A 2-ml volume of the urine sample was treated according to the procedure described above and a gas chromatogram is illustrated in Fig. 3. The retention time and the GC–MS pattern of peak X agreed completely with those of authentic anthranilamide. The amount present was determined to be 245 μg in 24-h urine.

The use of an electron capture detector for measuring anthranilamide at very low levels was investigated and, as is to be expected, a satisfactory response was obtained (Fig. 4).



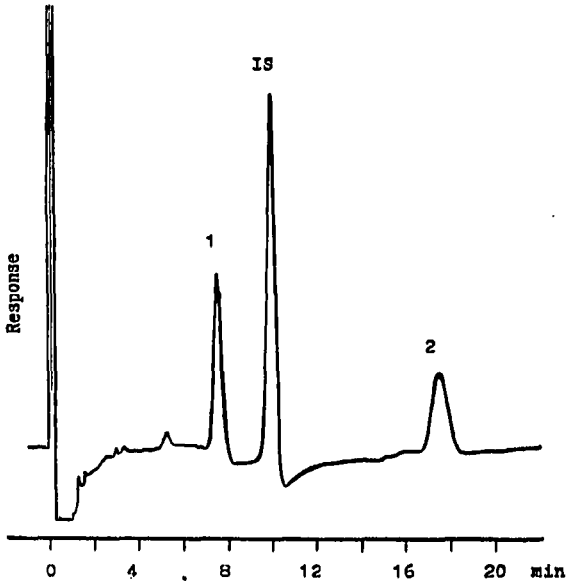


Fig. 3. GC of the urine sample of a rat administered with anthranilic acid. Conditions as in Fig. 1.

Fig. 4. Gas chromatogram of urinary anthranilamide. 1, A trifluoroacetyl derivative of anthranilamide; 2, ethyl N-trifluoroacetyl anthranilate. IS (internal standard), *p*-xylene dichloride. Conditions: 7% GE-XF 1105 on Gas-Chrom Z (80–100 mesh), 2.0 m × 3 mm I.D. glass column, 130°, ECD.

The removal of the excess of reagent was necessary because the presence of excess of reagent made satisfactory separations impossible. The trifluoroacetylated reaction mixture was evaporated for 5 min at reduced pressure and room temperature, diluted to an suitable volume with ethyl acetate and 1.0 μl of the solution was injected into the gas chromatograph. Calibration curves were linear in the range 0.1–0.4 μg of anthranilamide, and the recovery determined by carrying out three identical analyses was calculated to be 82.4 ± 2.5% (S.D.) for 0.4 μg of anthranilamide (Table II).

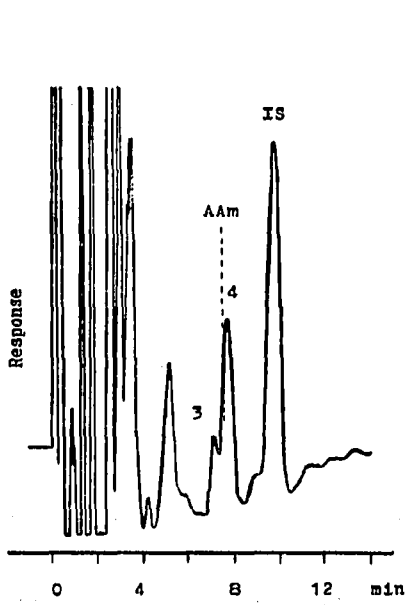
TABLE II

RECOVERY OF ADDED ANTHRANILAMIDE FROM AN AQUEOUS SOLUTION USING AN ELECTRON CAPTURE DETECTOR

Sample	*Taken* (μg)	*Found* (%)
1	0.400	80.7
2	0.400	81.9
3	0.400	84.8
Average		82.4

Fig. 4 shows a gas chromatogram obtained from the urine of the treated rat and peak 1 corresponds to an injection of about 0.2 μg of anthranilamide. No anthranilamide was detected in normal human urine by the HFID, but on a gas chromatogram obtained by using the ECD, two peaks appeared close to the retention time of an authentic sample, which had a response corresponding to about 0.13 μg per 10 ml of urine (Fig. 5). It is not evident whether peak 4, differing by 0.2 min in retention time, is due to the presence of anthranilamide in urine.

An interesting phenomenon is shown in Fig. 3. When the urine sample from the treated rat was shaken with 10 ml of chloroform in an acidic medium for the prior removal of some acidic substances before extracting anthranilamide in a basic medium, the height of peak Y was found to increase remarkably (shown by



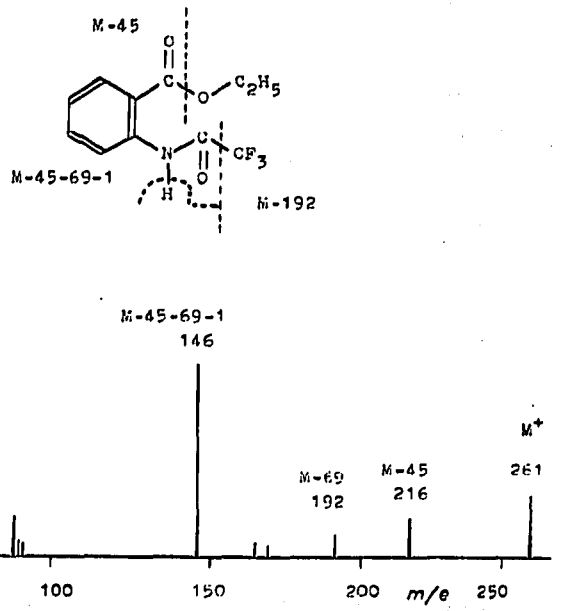


Fig. 5. GC of normal human urine. AAm = the retention time of trifluoroacetylated anthranilamide. Conditions as in Fig. 4.

Fig. 6. GC–MS spectrum of ethyl N-trifluoroacetyl anthranilate.

the dotted line in Fig. 3). In order to explain this phenomenon, an investigation of the structure of peak Y was performed by GC–MS, and its spectrum is illustrated in Fig. 6, which proves it to be ethyl N-trifluoroacetyl anthranilate. The retention time of peak Y agreed completely with that of authentic ethyl anthranilate.

It is known that anthranilic acid is excreted as conjugates with β-glucuronic acid or glycine on the carboxyl group[1], and the conjugated anthranilates are readily converted into anthranilamide in the presence of ammonia[6]. Commercial chloroform used for the extraction contains about 0.5% of ethanol as a stabilizer. In a manner similar to that with ammonia, it appears likely, therefore, that the exchange reaction with ethanol is readily effected. Further evidence of this effect was found by using ethanol-free chloroform or carbon tetrachloride for extraction, which resulted in the complete disappearance of peak Y, *i.e.* ethyl anthranilate. Now, it is evident that anthranilamide is formed from conjugated anthranilates by an exchange reaction with ammonia. The extent of the conversion increased rapidly with increasing ammonia concentration in the solution, and the following experiment was therefore carried out in order to determine whether the increase of anthranilamide might be caused by the addition of ammonia to normal human urine. A 10-ml volume of normal human urine was made alkaline with 1.2 ml of concentrated ammonia solution and allowed to stand overnight. The GC separation of the resultant sample solution was achieved by using an ECD, as shown in Fig. 7. The retention time was identical with that of an authentic sample, which indicates that peak 5 corresponds to anthranilamide produced by the addition of ammonia.

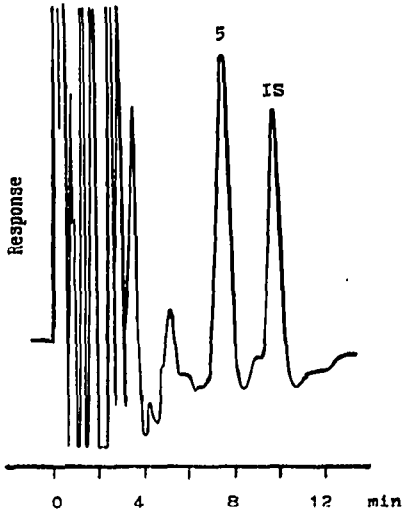


Fig. 7. GC of normal human urine allowed to stand overnight in concentrated ammonia solution. Conditions as in Fig. 4.

In conclusion, the results discussed above seem to indicate that anthranilamide is formed chemically from conjugated anthranilates by an exchange reaction with ammonia in blood or the bladder. It has been reported[1] that anthranilic acid injected into rats was metabolically converted into anthranilamide. However, insufficient

results were presented to support this conclusion. It would be desirable to carry out further investigations in order to establish with certainty whether urinary anthranilamide is an artifact product or a metabolic product of anthranilic acid.

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