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GAS CHROMATOGRAPHIC ANALYSIS OF TRYPTOPHAN METABOLITES

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SUMMARY

A procedure was developed for preparing the derivatives of tryptophan metabolites, which were classified into three groups; (A) kynurenine and its related compounds; (B) 2-aminoacetophenone and its related compounds; and (C) anthranilic acid and its related compounds. Groups A and B were converted to methoxy trifluoroacetylamino acetophenones and group C to methyl methoxy trifluoroacetylamino anthranilates. Each of these derivatives has proved to be useful for gas chromatographic separations using Apiezon grease L on Celite 545 SK with isothermal temperature.

INTRODUCTION

There have been intensive investigations of tryptophan metabolism. A number of metabolites of tryptophan have been shown to occur in the urine of various species under different conditions[1–24]. These metabolites have been isolated and identified by means of ion-exchange resin, paper and thin-layer chromatography. 3-Hydroxyanthranilic acid was studied by gas chromatography (GC) analysis by ROSE *et al.*[25]. However, there are few or no reports on the methods for GC analysis of the biologically important metabolites of tryptophan, including kynurenine, anthranilic acid, 2-aminoacetophenone and other related compounds. Therefore, attempts were made to prepare the derivatives of ten metabolites of tryptophan and to analyze the resulting derivatives by GC.

EXPERIMENTAL

Reagents

Anthranilic acid, 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid, DL-kynurenine, 3-hydroxy-DL-kynurenine and 5-hydroxy-DL-kynurenine were the same preparations as those used in previous studies[24,26]. 3,5-Dihydroxyanthranilic acid was generously provided by Dr. SHIRO SENOH, Institute for Food Chemistry, Osaka, Japan. 2-Aminoacetophenone, 2-amino-3-hydroxyacetophenone and 2-amino-5-hydroxyacetophenone were synthesized according to the methods described be-

fore[27–29]. Trifluoroacetic anhydride was obtained from Tokyo Chemical Industry Co. Ltd. Etherial diazomethane was prepared from N-methyl-N-nitrosotoluene-*p*-sulphonamide (Tokyo Chemical Industry Co. Ltd.). It remained stable at least for 3 weeks when stored over potassium hydroxide pellets at −10°.

Apparatus and working conditions

A Hitachi–Perkin-Elmer Model F-6 gas chromatograph with a flame ionization detector was used. The 6 ft. × 3 mm I.D. stainless-steel column used was packed with 10% (w/w) Apiezon grease L on Celite 545 SK (Nippon Chromatographic Industry Co. Ltd.). The column oven temperature was 230°, the detector and injection port temperatures, 270° and 250°, respectively. The gas flow rates of nitrogen, hydrogen and air were 70, 40 and 300 ml/min, respectively. The sample size was 2.0 μl. The chart speed was 1 cm/min.

Preparation of derivatives

Eleven tryptophan metabolites were classified into three groups. Each group is designated through the text of this paper as Group A, B or C. Group A contained DL-kynurenine, 3-hydroxy-DL-kynurenine and 5-hydroxy-DL-kynurenine. Group B contained 2-aminoacetophenone, 2-amino-3-hydroxyacetophenone and 2-amino-5-hydroxyacetophenone. Group C contained anthranilic acid, 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid and 3,5-dihydroxyanthranilic acid. Each group was a mixture of equal amounts (2 mg) of its respective components. Groups B and C were quantitatively converted to methoxy N-trifluoroacetyl derivatives and methyl methoxy N-trifluoroacetyl derivatives, respectively as follows. The mixture of Group B or Group C was placed in a glass-stoppered test tube with 1 ml of etherial diazomethane and left overnight in the dark at room temperature. After the solvent was removed under a stream of nitrogen gas *in vacuo*, 0.5 ml of chloroform and 0.2 ml of trifluoroacetic anhydride were added. The tube was sealed and allowed to stand in the dark at room temperature at least for 30 min. The solution was evaporated to dryness under a stream of nitrogen gas *in vacuo*. The dry residue was dissolved in a small volume of anhydrous ethyl acetate for GC examination.

DL-Kynurenine, 3-hydroxy-DL-kynurenine and 5-hydroxy-DL-kynurenine in Group C were converted to 2-aminoacetophenone, 2-amino-3-methoxy-acetophenone and 2-amino-5-methoxyacetophenone, respectively, as follows. The mixture of Group C was dissolved in 2 ml of methanol, and 4 ml of etherial diazomethane were added. After the solution stood overnight in the dark at room temperature, the solvent was removed under nitrogen *in vacuo*. The residue was heated for 1 h with 2 ml of 1 *N* HCl to hydrolyze the methyl esters on the boiling water bath. After the resulting yellow-brown solution was adjusted to pH 7.0 with 6 *N* NaOH, 0.2 ml of 0.1 *N* $Ba(OH)_2$ was added, and the solution was refluxed for 1 h at 100°. The solution was refluxed with 2.5 ml of 2 *N* NaOH for 1 h at 100°, and then was adjusted to pH 7.0 with 4 *N* HCl and shaken with three 30-ml portions of diethyl ether. The combined ether extract was evaporated to dryness *in vacuo*. The resulting methoxy aminoacetophenones were trifluoroacetylated by the procedure described above. The resulting derivatives were dissolved in a small volume of ethyl acetate for GC analysis.

RESULTS AND DISCUSSION

Eleven tryptophan metabolites were classified into three groups: (A) kynurenine and its related compounds; (B) 2-aminoacetophenone and its related compounds; (C) anthranilic acid and its related compounds, because each group can be isolated according to the procedure of ROY AND PRICE[30]. Groups B and C were quantitatively converted to methoxy N-trifluoroacetyl derivatives and methyl methoxy N-trifluoroacetyl derivatives respectively, by treating with diazomethane overnight and trifluoroacetic anhydride for 30 min at room temperature as described under EXPERIMENTAL. All the derivatives were stable under anhydrous condition at least for one week at room temperature. In the previous investigation, the resolution of two methoxy N-trifluoroacetyl derivatives of 2-amino-3-hydroxyacetophenone and 2-amino-5-hydroxyacetophenone in Group B, or two methyl methoxy N-trifluoroacetyl derivatives of 3-hydroxyanthranilic acid and 5-hydroxyanthranilic acid in Group C were not satisfactory using Silicone XE-60 on Diasolid (Nippon Chroma-

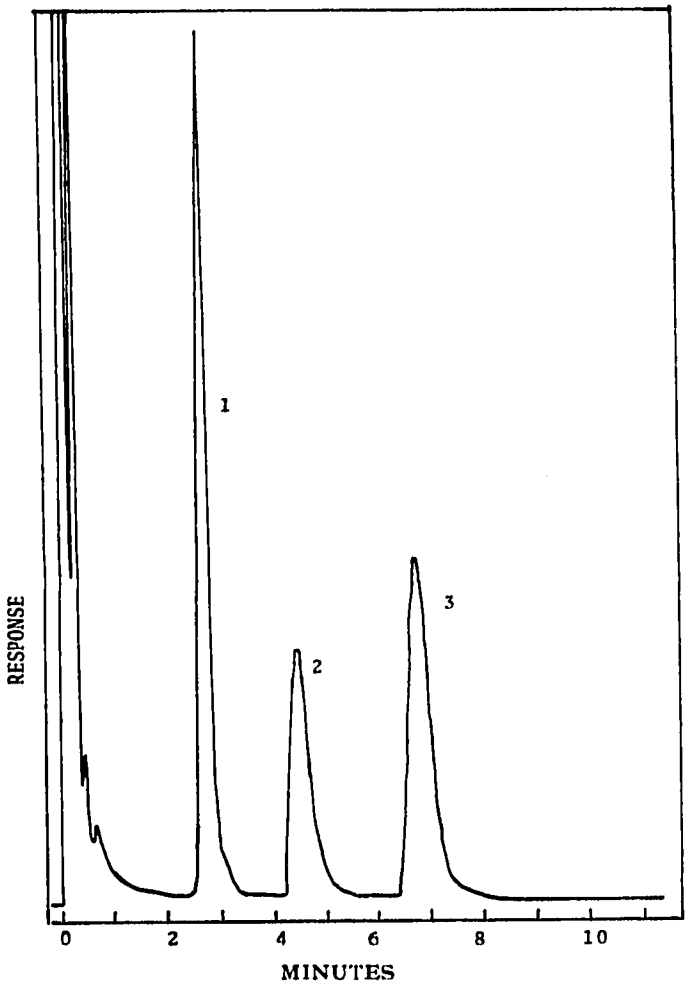

Fig. 1. Gas chromatogram of the derivatives of anthranilic acid and its related compounds. Working conditions are as described in the text. 1 = methyl 2-trifluoroacetylamino anthranilate; 2 = methyl 3-methoxy-2-trifluoroacetylamino anthranilate; 3 = methyl 5-methoxy-2-trifluoroacetylamino anthranilate; 4 = methyl 3,5-dimethoxy-2-trifluoroacetylamino anthranilate.

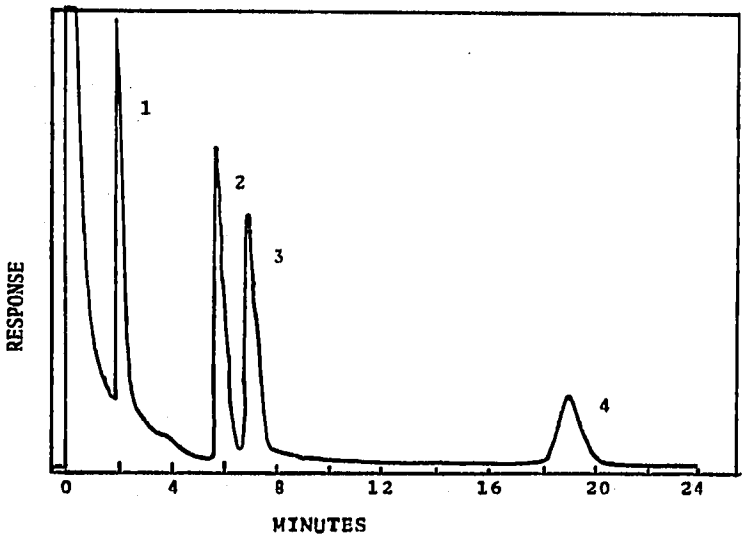

Fig. 2. Gas chromatogram of the derivatives of 2-aminoacetophenone and its related compounds. Working conditions are as described in the text. 1 = 2-trifluoroacetylamino acetophenone; 2 = 3-methoxy-2-trifluoroacetylamino acetophenone; 3 = 5-methoxy-2-trifluoroacetylamino acetophenone.

tographic Industry Co. Ltd.)[28]. In this work, the clear separation of these compounds was obtained by using Apiezon grease L on Celite 545 SK with isothermal temperature. The chromatogram of the derivatives from the mixture of the members, belonging to Group B is illustrated in Fig. 1. That of Group C is shown in Fig. 2. As shown in both figures, the peaks with good shapes characterized by specific retention time were obtained for each component. The separation was complete and no extraneous peaks were observed. Working conditions are described under EXPERIMENTAL. The use of several other conditions resulted in unsatisfactory separation. In previous experiments, we failed to prepare the volatile derivatives of Group A (kynurenine, 3-hydroxykynurenine and 5-hydroxykynurenine) by treating with diazomethane and trifluoroacetic anhydride. Subsequently, it was attempted to convert each component of Group A to the corresponding methoxy aminoacetophenones. This attempt was successful. The yield of each product was found to be complete from its molar absorption coefficient in ethyl alcohol (2-aminoacetophenone, $\varepsilon_{364\ m\mu} = 3500$; 2-amino-3-methoxyacetophenone, $\varepsilon_{370\ m\mu} = 3750$; 2-amino-5-methoxyacetophenone, $\varepsilon_{390\ m\mu} = 3800$). The methoxy aminoacetophenones were trifluoroacetylated prior to GC analysis. The retention times of three derivatives prepared were the same as those of trifluoroacetylamino acetophenone, 3-methoxy-2-trifluoroacetylamino acetophenone and 5-methoxy-2-trifluoroacetylamino acetophenone, respectively. Other important tryptophan metabolites, quinoline compounds such as kynurenic acid, xanthurenic acid, 6-hydroxykynurenic acid, quinaldic acid, 4-hydroxyquinoline, 4,6-dihydroxyquinoline, 4,8-dihydroxyquinoline and others were also subjected to GC analysis. The separation of these metabolites was not satisfactory because of the difficulty of quantitative conversion of these compounds to volatile derivatives. The GC analysis of quinoline compounds and determination of tryptophan metabolites are in progress.

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