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Gas Chromatography of Urinary Anthranilic Acid and 3-Hydroxyanthranilic Acid by Solvent Extraction Method

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A simple procedure has been developed for the simultaneous determination of the urinary anthranilic and 3-hydroxyanthranilic acids by gas chromatography. The method is based on extracting from urine with chloroform or ether at pH 3.0, followed by converting to their trifluoroacetyl derivatives. A clear separation of anthranilic and 3-hydroxyanthraniclic acids was achieved by using 3% OV-17 column at 110°. Compared with the recovery values of anthranilic acid, those of 3-hydroxyanthranilic acid are considerably low, which is due to the decomposition of 3-hydroxyanthranilic acid in the extraction procedure.

Anthranilic acid (AA) and 3-hydroxyanthranilic acid (3-OH-AA) are formed as intermediates in the metabolism of tryptophan to nicotinic acid. In a previous paper,[2] the urinary AA was converted to salicylic acid with nitrous acid and was methylated with diazomethane and then analyzed by gas chromatography. This method was somewhat cumbersome and 3-OH-AA could not be determined. In the present paper, a simpler method for the determination of AA and a procedure for the simultaneous determination of AA and 3-OH-AA are described. This procedure is based on extracting with chloroform or ether at pH 3.0, trifluoroacetylation, and then separation by gas chromatography. This techniques has been applied to assay the urinary AA and 3-OH-AA.

Experimental

Apparatus and Conditions——A Shimadzu Model GC-4APF gas chromatograph equipped with a hydrogen flame ionization detector (HFID) and a Shimadzu Model GC-4APE gas chromatograph equipped with an electron capture detector (ECD) were used. The 1.5 m × 3 mm I.D. stainless steel column and glass column were packed with 3% OV-17 on Gas Chrom Z (80—100 mesh), respectively. The column temperature was 110°. Nuclear magnetic resonance (NMR) Spectra: A Hitachi NMR spectrometer Model R-20B was used. The spectra were measured in $CDCl_3$. Infrared (IR) Spectra: A JASCO DS-403G grating infrared spectrophotometer was used. The spectra were measured in KBr disc.

Method A: Determination of AA: The pH of a 20 ml of sample solution containing more than 20 μg of AA was adjusted to 3.0 with KOH solution by using a pH meter and the solution was saturated with NaCl, followed by extracting with two 5 ml portions of $CHCl_3$. To the combined $CHCl_3$ extracts, a few drops of dimethoxypropane (DMP) was added, and then the mixture was evaporated to dryness under reduced pressure. To the residue, a drop of ethyl acetate and 0.25 ml of trifluoroacetic anhydride (TFAA) were added and the mixture solution was allowed to stand for 10 min. Then as an internal standard, an appropriate volume of α,α'-dichloro-*p*-xylene 200 μg/ml in ethyl acetate was added and 1 μl was injected onto the gas chromatograph. On the other hand, a standard solution of AA in ethyl acetate (10 mg/10 ml) was evaporated to dryness at room temperature under reduced pressure and a calibration curve was prepared by plotting AA concentration *vs.* peak heigt ratio to an internal standard. Urine samples were diluted 1:1 with 0.1 N HCl and were applied to the determination through the entire procedure described above.

Method B: Simultaneous Determination of AA and 3-OH-AA: A 20 ml of sample solution containing more than 20 μg of each AA and 3-OH-AA was adjusted to pH 3.0 with KOH solution. The solution was saturated with NaCl, and then extracted with two 5 ml portions of peroxide-free ether. The combined ether extracts were dried by adding 1 ml of DMP and then treated by the same procedure as Method A.

1) Location: *Mitahora, Gifu.*
2) K. Hirano, K. Mori, S. Kawai and T. Ohno, *J. Chromatog.*, **64**, 174 (1972).

Calibration curves were prepared from the standard solution of AA in $CHCl_3$ (4 mg/10 ml) and 3-OH-AA in MeOH (4 mg/50 ml) according to the procedure in Method A. Urine samples were applied after dilution (1→2) with 0.1N HCl.

Preparation of Trifluoroacetyl (TFA) Derivatives of AA and 3-OH-AA——Excess TFAA was added to 100 mg of pure AA in ethyl acetate and the mixture was washed by shaking vigorously with cold water and $CHCl_3$. The $CHCl_3$ solution was dried over Na_2SO_4 and evaporated to dryness. mp 47—49°. *Anal.* Calcd. for $C_9H_4O_2NF_3$: C, 50.24; H, 1.88. Found: C, 50.38; H, 2.14. IR ν_{max}^{KBr} cm^{-1}: 1780 (C=O), 1670 (C=N). NMR δ_{ppm} in $CDCl_3$: 7.65—8.32 (aromatic protons). TFA derivative of 3-OH-AA was prepared by the similar procedure as that of AA. mp 97—99°. *Anal.* Calcd. for $C_{11}H_3O_4NF_6$: C, 40.38; H, 0.93. Found: C, 40.61; H, 1.12. IR ν_{max}^{KBr} cm^{-1}: 1810 and 1790 (2C=O), 1675 (C=N). NMR δ_{ppm} in $CDCl_3$: 7.70—8.32 (aromatic protons).

Result and Discussion

Rose and Toseland[3] reported a gas chromatographic separation of the urinary 3-OH-AA as methyl N-acetyl-3-methoxy anthranilate. Noguchi, *et al.*[4] prepared methyl methoxy N-TFA and methyl N-TFA derivatives for gas chromatographic separation of AA and 3-OH-AA. In their procedure, it was necessary for the reaction with ethereal diazomethane to proceed overnight. In the results of our preliminary examinations on the preparation of various derivatives for separation by gas chromatography, AA and 3-OH-AA reacted easily with TFAA and each product gave a single, sharp peak. A chromatogram of TFA derivatives of AA and 3-OH-AA was shown in Fig. 1 by using 3% OV-17, at 110°.

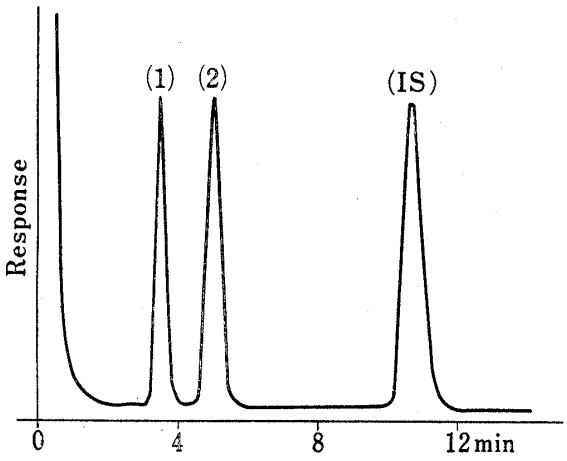

Fig. 1. Separation of (1) Anthranilic Acid and (2) 3-Hydroxyanthranilic Acid as Their Trifluoroacetyl Derivatives Using HFID as a Detector

IS: an internal standard (α,α'-dichloro-*p*-xylene)
3% OV-17, 1.5 m, stainless steel column, 110°

Table I. Recovery of Anthranilic Acid

	Taken (μg)	Found (%)
1	100	98.8
2	100	95.0
3	100	97.7
4	100	97.7
av.		97.4
5	20	100.0
6	20	100.0
7	20	107.5
av.		102.5

Suitable conditions for extraction of AA and 3-OH-AA in aqueous solutions were as follows.

A) Optimum pH is 3.0.

B) Effective organic solvents for extraction of AA are chloroform and ether, and for 3-OH-AA is only ether.

The procedure for gas chromatographic separation of only AA is described in Method A in the Experimental section. Chloroform is the most suitable solvent for extraction of AA, because the distribution coefficient for AA between chloroform and water is high enough

3) D.P. Rose and P.A. Toseland, *Clinica Chimica Acta*, **17**, 235 (1967).
4) T. Noguchi, H. Kaseda, N. Konishi and R. Kido, *J. Chromatog.*, **55**, 291 (1971).

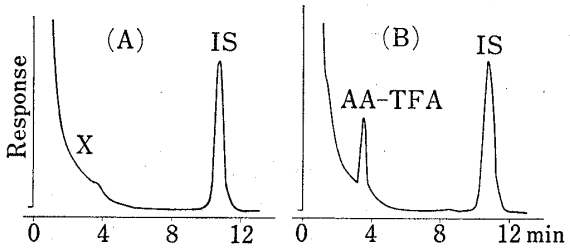

Fig. 2. Chromatogram of Samples from a Normal Urine (A) and a Spiked Urine (B) to Which 50 μg of Anthranilic Acid was Added AA-TFA: Anthranilic Acid Trifluoroacetyl Derivative

IS: α,α′-dichloro-*p*-xylene
3% OV-17, 1.5 m, stainless steel column, 110°, HFID

Table II. Recovery of Anthranilic Acid from Spiked Control Urine by Method A

No	Added (μg)	Found (%)
1	50	80.1
2	50	83.8
3	50	84.5
4	50	86.3
av.		83.7
5	20	95.2
6	20	84.1
7	20	92.1
8	20	79.4
av.		87.7

for quantitative extraction and after shaking the chloroform layer is separated clearly from the aqueous layer. The results obtained through the entire procedure from the standard solutions containing 100 μg and 20 μg of AA show good recovery (97.4% and 102.5%) as given in Table I. AA in a normal human urine was assayed by Method A (Fig. 2A). A small shoulder X at 3.5-minute retention time agreed with that of the TFA derivative of AA. For the recovery studies, control urine samples were spiked with 50 μg and 20 μg of pure AA. The height of shoulder X (namely, peak AA-TFA in Fig. 2B) was found to increase in proportion to the amount of added AA. A typical gas chromatogram is seen in Fig. 2B and results are given in Table II. An effective solvent is only ether for the simultaneous extraction of AA and 3-OH-AA and quantitative extraction is effected by shaking with two 5 ml portions of ether at pH 3.0 from aqueous solution. However, there exist certain disadvantages to this procedure. One of them is that 3-OH-AA is unstable in ether. Therefore, in order to minimize the decomposition, peroxide-free ether must be used as described by Rose,[3] which is prepared by passing through alumina column. Another disadvantage is that a small amount of water comes into the ether layer, which disturbs evaporation step prior to preparation of TFA derivatives. Addition of large amount of sodium sulfate anhydride for dehydration causes loss of the experimental compound. DMP has been reported to be a good drying agent.[5–8] DMP reacts with water rapidly in a slightly acid environ-

Table III. Recovery of Anthranilic Acid (AA) and 3-Hydrocyanthranilic Acid (3-OH-AA) from Spiked Control Urine by Method B

	Taken AA (μg)	Found (%)	Taken 3-OH-AA (μg)	Found (%)
1	200	103.0	240	66.0
2	200	108.8	240	65.4
3	200	109.0	240	65.3
4	200	107.4	240	65.3
5	200	109.3	240	65.0
av.		107.5		65.4
6	100	90.5	120	62.0
7	100	90.8	120	62.2
8	100	90.8	120	62.4
9	100	90.6	120	62.6
10	100	90.8	120	62.3
av.		90.7		62.3

5) D.S. Erley, *Anal. Chem.*, **29**, 1564 (1957).
6) F.E. Critchfield and E.T. Bishop, *Anal. Chem.*, **33**, 1034 (1961).
7) J.H. Martin and A.M. Knevel, *J. Pharm. Sci.*, **54**, 1464 (1965).
8) N.Y. Mary, *J. Chromatog.*, **42**, 411 (1969).

ment (pH<6) to form methanol and acetone. In our work, DMP was found to be an excellent reagent for the removal of water as shown by the following facts.

A) The reaction of DMP with water is practically instantaneous at room temperature.

B) The products, methanol and acetone as well as the excess DMP (bp=79°), are volatile and evaporated readily to dryness at room temperature.

C) DMP does not disturb the quantitative separation of the compounds.

According to Method B, the recovery test was carried out from the standard solution containing both of AA and 3-OH-AA, and the results are given in Table III. A gas chromatographic separation for more sensitivity was achieved successfully by using ECD and a glass column as shown in Fig. 3. A calibration curve showed a straight line for the assay of 1.6—4.8 μg/ml concentrations of AA and 3-OH-AA, and the recovery values obtained by Method B are shown in Table IV. As can be seen from the recovery studies, the recovery values of 3-OH-AA are considerably low, compared with those of AA. It appears to be difficult to prevent completely the decomposition of 3-OH-AA in the above extraction procedure. Now, the use of ion exchange resin is being investigated in an effort to increase the recovery of 3-OH-AA from urine.

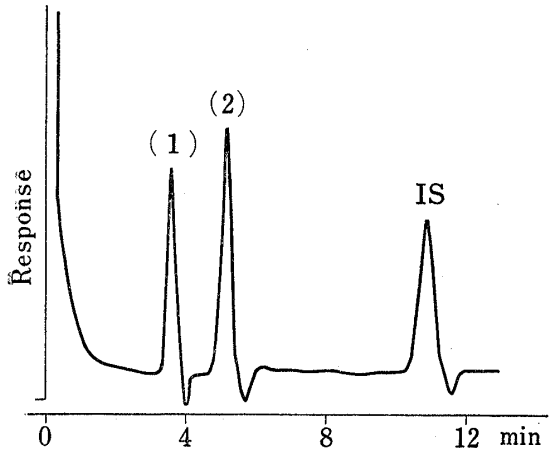

Fig. 3. Separation of (1) Anthranilic Acid and (2) 3-Hydroxyanthranilic Acid as Their Trifluoroacetyl Derivatives Using ECD as a Detector

IS: α,α′-dichloro-*p*-xylene
3% OV-17, 1.5 m, glass column, 110°

Table IV. Recovery of AA and 3-OH-AA (ECD)

	AA		3-OH-AA	
	Taken(μg)	Found(%)	Taken(μg)	Found(%)
1	4.0	83.5	4.8	62.3
2	4.0	80.0	4.8	62.3
3	4.0	67.1	4.8	61.5
av.		77.0		62.0

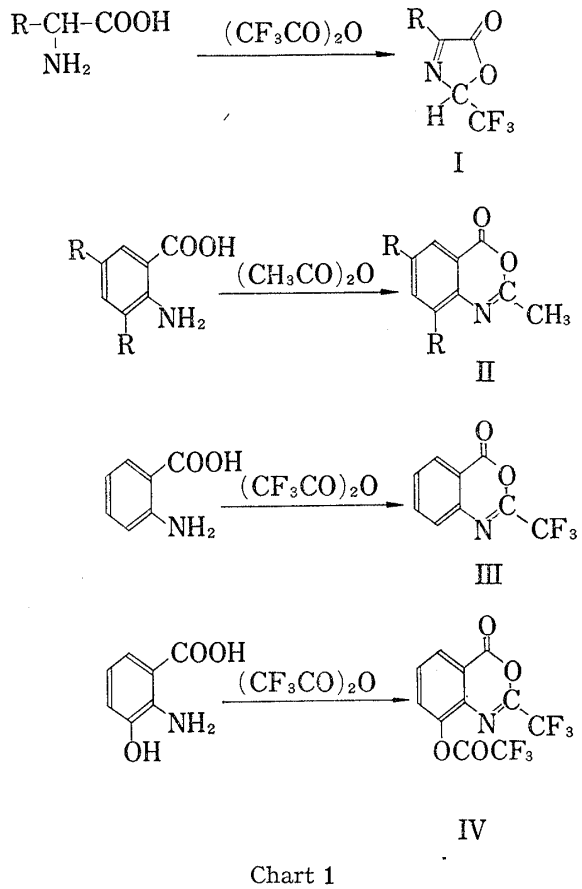

Chart 1

The Structures of TFA-Derivatives of AA and 3-OH-AA

α-Amino acid has been reported to form 2-trifluoromethyl-4-substituted-3-oxazolin-5-one (I in Chart 1) by reaction with TFAA.[9] The structures of AA and 3-OH-AA are quite similar to that of α-amino acid, therefore it is reasonable to presume that a reaction with TFAA give the derivative with the similar structure as that of α-amino acid. The work of Parmar

9) F. Weygand, W. Steglich and H. Tanner, *Liebig's Ann. Chem.* **658**, 128 (1962).

and Arora[10] supports this speculation where the corresponding 2-methyl-3-(4-benzhydrazide)-4-quinazolones (II) were synthesized from reaction of substituted anthranilic acids with acetic anhydride. From NMR, IR spectra and elementary analysis the structures of TFA derivatives of AA and 3-OH-AA are concluded to be 2-trifluoromethyl-4H-3,1-benzoxazin-4-one (III) and 2-trifluoromethyl-8-trifluoroacetoxy-4H-3,1-benzoxazin-4-one (IV) respectively. The NMR spectra of them show only aromatic protons as listed in Experimental section and the signals at 1670 cm^{-1} and 1675 cm^{-1} in IR spectra suggest existence of $\nu_{C=N}$ respectively. The signal at 1780 cm^{-1} means $\nu_{C=O}$ in compound III and two signals at 1790 cm^{-1} and 1810 cm^{-1} correspond to two $\nu_{C=O}$ in compound IV.

Acknowledgement The authers are grateful to Dr. A. Tsuji for measurement of NMR spectra.

10) S.S. Parmar and R.C. Arora, *J. Med. Chem.*, **10**, 1182 (1967).