CHROM. 6067

COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY IN THE DETERMINATION OF URINARY ANTHRANILIC ACID

KAZUYUKI HIRANO, MASAHARU NARUSE, SATOSHI KAWAI AND TAKEO OHNO
Gifu College of Pharmacy, Mitahora, Gifu (Japan)
(Received February 29th, 1972)

SUMMARY

Urinary anthranilic acid was converted to methyl salicylate by treatment with nitrous acid and diazomethane, and was analysed by gas chromatography using an OV-17 column. The peak having a retention time the same as that of authentic methyl salicylate was investigated by combined gas chromatography–mass spectometry and was proved to consist of two components, anthranilic acid and phenylacetic acid. Furthermore, from the consideration of gas chromatography–mass spectrometry results, it was found that a large amount of *p*-cresol was excreted in normal urine. A satisfactory separation of the urinary anthranilic acid, phenylacetic acid and *p*-cresol was achieved by using a mixed column of OV-17 and XF-1105.

INTRODUCTION

Anthranilic acid is formed as one of the tryptophan metabolites and it has been reported that an increase in a amount of the urinary anthranilic acid is associated with some diseases. In a previous paper[1], a procedure was developed for the gas chromatographic determination of the urinary anthranilic acid where the anthranilic acid was converted to methyl salicylate with nitrous acid and diazomethane, and a single peak X obtained from a normal urine was shown to have the same retention time as that of methyl salicylate (Fig. 1).

There has been no report dealing with the gas chromatographic determination of the urinary anthranilic acid, and therefore the identification of this interesting peak X was tried by combined gas chromatography–mass spectrometry (GC–MS). In the results, it became clear that the peak X consisted of two compounds, anthranilic acid and phenylacetic acid, and a complete separation of both components was achieved by using a mixed column of OV-17 and XF-1105. In addition, an interesting phenomenon was found that a large amount of *p*-cresol is excreted in normal urine.

EXPERIMENTAL

Apparatus and conditions

A Shimadzu, Model GC-4APF, gas chromatograph equipped with a hydrogen flame ionization detector (HFID) was used, and the 3.0 m × 3 mm I.D. stainless-steel column was packed with 10 % XF-1105 after 5 ml of 4 % OV-17. The column oven

temperature was 130° and the detector and injection temperatures were both 150°. The gas flow-rates of nitrogen, hydrogen and air were 30, 65 and 1000 ml/min, respectively. The sample size was 1.0 μl and the chart speed was 0.5 cm/min.

Gas chromatography-mass spectrometry

The mass spectra of methyl salicylate, methyl phenylacetate, *p*-cresol and the experimental peak obtained from urine samples were measured on a Model RMS-4 Hitachi mass spectrometer. The operating conditions were: ion acceleration potential, *m/e*, 1.5 kV; chamber potential, 70 eV; total emission, 80 μA; target current, 60 μA. All samples were introduced into the ionization chamber through a Hitachi Model K-53 gas chromatograph. A 1.0-m stainless-steel column packed with 2% OV-17 on Gas-Chrom Z (80–100 mesh) was used, and the temperature was maintained at 100°. The molecular separator was contained in the GC oven. The inlet line to the mass spectrometer source was kept at 120°. For a given GC peak, a single accurate mass measurement could be made on a compound passing through the system.

Standard procedure

A 10-ml volume of urine was diluted with 1 ml of concentrated HCl and with 0.1 *N* HCl to 20 ml. To the solution obtained, 1 ml of 0.2 *M* $NaNO_2$ was added, then the mixture was allowed to stand for 20 min in an ice-bath. After this period of time, excess HNO_2 was decomposed by adding 1 ml of 0.2 *M* sulfamic acid, followed by heating the solution on a water-bath at 80° under reflux for 35 min to hydrolyse the diazonium salt produced. After cooling in an ice-bath, a 0.5-ml aliquot of a chloroform solution containing a known amount of phenylpropionic acid (as internal standard) was added and then the mixture was extracted with 5-ml portions of chloroform. To the combined chloroform extracts, 0.5 ml of dimethoxypropane was added as a dehydrating agent and the solution mixture was evaporated just to dryness at room temperature at a reduced pressure, and the residue was methylated with freshly prepared ethereal diazomethane. The solution was finally concentrated and injected into the gas chromatograph.

RESULTS AND DISCUSSION

The conditions for the standard procedure were discussed in detail in a previous paper[1]. Dimethoxypropane was used as an excellent reagent for the dehydration of the organic solvent. A gas chromatogram obtained from a urine sample is illustrated in Fig. 1. The separation was carried out at 100° for mass spectrometry. The peak X in Fig. 1 has a retention time the same as that of methyl salicylate and its GC–MS pattern is shown in Fig. 2A. In Fig. 2A, the prominent ions with *m/e* of 152, 121, 120 and 92 have the same relative intensities as those of authentic methyl salicylate (Fig. 2B), which means that the peak X corresponds to methyl salicylate derived from the urinary anthranilic acid. However, the additional signals with *m/e* of 150, 108, 107 and 91 suggest the presence of co-existing components, and a further investigation was made to identify these fragmentation patterns. After treatment with nitrous acid, the compound extractable with chloroform in an acidic medium from urine samples did not show any peaks at the retention time of the peak X, unless it was methylated with diazomethane, which suggests that the

co-existing component contains a carbonyl group, –COOH, in the structure. The ion at *m/e* 150 indicates the molecular weight, and the presence of a benzyl substituent $C_6H_5CH_2$– can be inferred by the presence of an ion at *m/e* 91. That is, methyl phenylacetate is considered to lose the methyl carbonate from the molecular ion as the

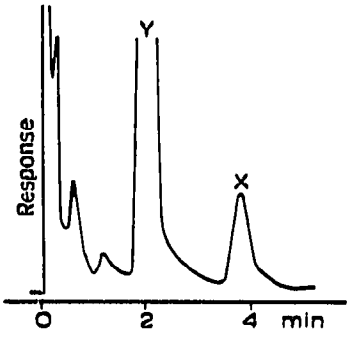

Fig. 1.

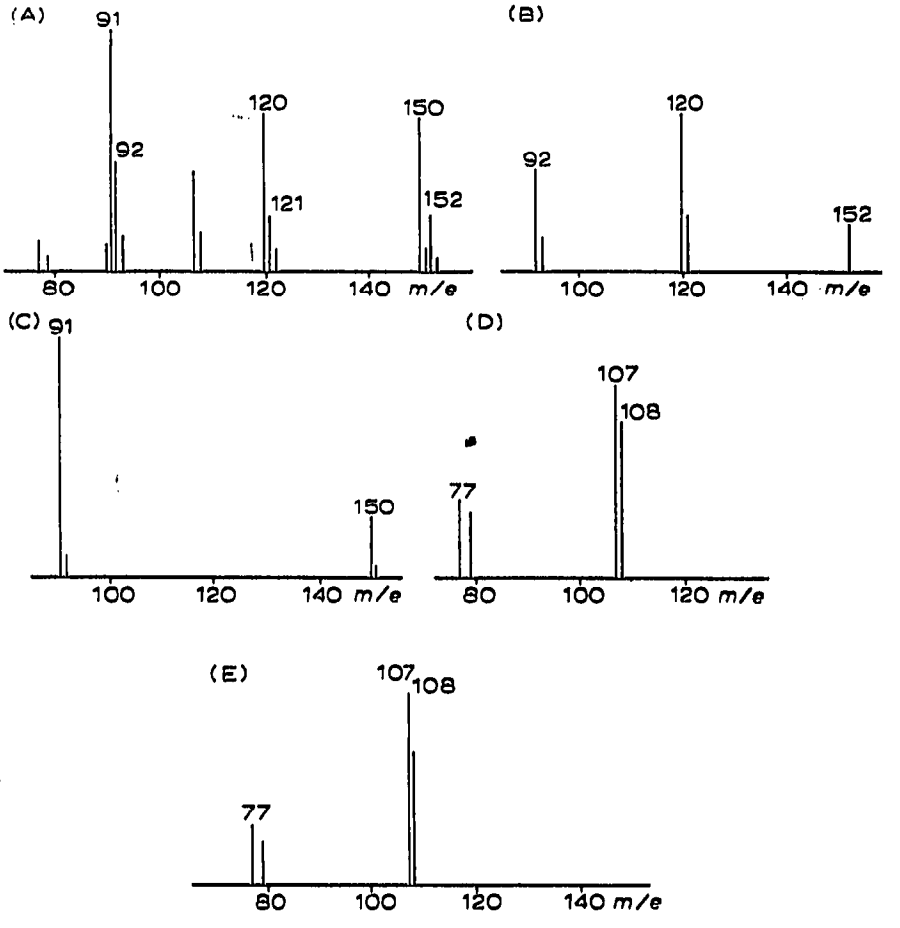

Fig. 2.

Fig. 1. Gas chromatographic separation of the urinary anthranilic acid by the nitrous acid method. 2% OV-17, 1-m stainless-steel column, 100°, FID.

Fig. 2. GC–MS spectra. A, peak X in Fig. 1; B, authentic methyl salicylate; C, authentic methyl phenylacetate; D, peak Y in Fig. 1; E, authentic *p*-cresol.

neutral olefin, to give the base peak of the spectrum, at *m/e* 91, and this theory was supported conclusively by the agreement with the GC–MS spectrum of authentic methyl phenylacetate (Fig. 2C). For a gas chromatographic separation of methyl salicylate (derived from anthranilic acid) and methyl phenylacetate, a mixed column of 10% XF-1105 + 4% OV-17 was successfully used and, as expected, a satisfactory separation of the peak X was achieved as shown in Fig. 3. OV-17 was used to avoid the disturbance by other co-existing components in urine. Phenylacetic acid is one of the normally occurring urinary metabolites of phenylalanine. It is considered to be excreted as conjugated forms, for unless the urine sample is heated to hydrolyse it, its gas chromatographic peak does not appear. Next, the explanation of the *m/e* 108 and 107 peaks was considered. The relative intensities of the *m/e* 108 and 107 peaks in the mass spectrum of Fig. 2A agree with those of the peak Y in Fig. 1

(Fig. 2D), which indicates the contamination of the peak X by tailing of the peak Y component. Now, the peak Y appeared whether or not treatment with diazomethane was used, which suggests that the peak Y component does not contain a carboxyl group, –COOH. In addition, the peak Y is also considered to be excreted as its

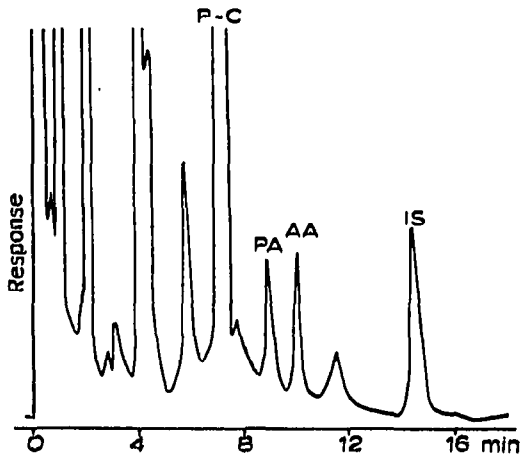

Fig. 3. Gas chromatographic separation of a normal urine sample. 10% XF-1105 + 4% OV-17, 3-m stainless-steel column, 130°, FID. P-C = *p*-cresol; PA = methyl phenylacetate; AA = methyl salicylate; IS = internal standard (methyl phenylpropionate).

TABLE I

DETERMINATION OF ANTHRANILIC ACID (AA), PHENYLACETIC ACID (PA) AND *p*-CRESOL (P-C) IN HUMAN URINE

Sample No.	*Sex*	*Age*	*Before (A) or after (B) child-bearing*	*AA (mg/day)*	*AA/PA*	*P-C*
1	F		A	14.7	3.21	
2	F		A	6.00	5.43	
3	F		A	8.21	1.87	++
4	F		A	2.75	0.58	++
5	F		A	1.07	0.69	±
6	F		A	5.39	2.44	++
7	F		A	5.82	0.58	++
8	F		A	1.25	3.50	—
9	F		B	7.52	2.29	—
10	F		B	2.76	0.50	—
11	F		B	3.62	1.37	—
12	F		B	2.36	0.65	—
13	M	22		11.5	1.35	++
14	M	24		2.13	0.63	++
15	M	22		9.06		++
16	M	3		1.89	0.21	±
17	M	2		5.58	0.58	++
18	M	43		6.84	0.89	++
19	F	21		2.04	2.75	++
20	F	22		6.32	0.72	++
21	F	21		2.97	2.67	++
22	F	22		5.67	1.17	++
23	F	22		2.30	0.29	++
24	F	3		2.25	9.60	±
25	F	2		5.10	1.25	++

conjugate, from the fact that only when it is hydrolysed does it appear on a gas chromatogram. The fragmentation pattern of *m/e* 77, 78 and 79 in Fig. 2D suggests the presence of the benzene skeleton, and *m/e* 108 suggests the molecular weight. Therefore, from these results, it appears that the peak Y is cresol or benzyl alcohol, and conclusive evidence was obtained that it is *p*-cresol from the agreement of the retention time and the mass spectrum with those of authentic *p*-cresol (Fig. 2E). Both mass spectra, Fig. 2D and 2E, were completely identical. Thus, the simultaneous determination could be carried out under the same gas chromatographic conditions for anthranilic acid, phenylacetic acid and *p*-cresol in urine samples. Anthranilic acid has been known as vitamin L, lactation factor, so in order to see if changes in the amount of urinary anthranilic acid are correlated with child-bearing, the urinary anthranilic acid of women before and after child-bearing were assayed by this technique and the results were compared with those for normal subjects. As can been seen from the results in Table I, an increase in the amount of the urinary anthranilic acid is not associated with any of child-bearing, age and sex. The ratio of anthranilic acid to phenylacetic acid also shows no correlation with any of them. However, an interesting fact was found that *p*-cresol was not excreted in the urine of women after child-bearing. *p*-Cresol has been known to be excreted as one of the metabolites of tyrosine via *p*-hydroxyphenylacetic acid in the micro-organism *Escherichia coli phenolgenes*. From the results of our preliminary experiments, it appears likely that the administration of the antibiotic, albiocin, is responsible for the disappearance of the urinary excretion of *p*-cresol after child-bearing. However, it has been a serious point of discussion[2] as to what the human urinary *p*-cresol can be attributed, and it would be desirable to carry out further investigations. Further investigations are currently being performed using germ-free animals to decide the origin of the human urinary *p*-cresol and its biological significance, which will be the subject of a future communication.

ACKNOWLEDGEMENTS

We are grateful to Mr. A. TAKEDA (Laboratory of Racing Chemistry) for measuring the GC-MS spectra. Urine samples of women before and after child-bearing were kindly supplied by Dr. K. NAGAI, for which we express our appreciation.

REFERENCES

1 K. HIRANO, K. MORI, S. KAWAI AND T. OHNO, *J. Chromatogr.*, 64 (1972) 174.
2 G. SUGANO AND R. MINAKUCHI, *Saishinigaku*, 14 (1959) 924.