Journal of Chromatography, 166 (1978) 294–298

CHROM. 11,249

Note

Electron-capture gas chromatography of phenolic acids as O-pentafluorobenzoyl methyl esters

MASAMI MAKITA, SHIGEO YAMAMOTO and KAZUKO ONO
Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama (Japan)
and
TOYOSHI KATAGI
Faculty of Pharmaceutical Sciences, Mukogawa University, 4-16 Edagawa, Nishinomiya, Hyogo (Japan)
(Received May 22nd, 1978)

Ultramicro-analysis of a variety of phenols by electron-capture gas chromatography (GC–ECD) of the conveniently prepared pentafluorobenzoyl (PFB) derivatives has been reported[1]. The preparation of the PFB derivatives was carried out in an aqueous alkaline medium by treating the compounds with pentafluorobenzoyl chloride at room temperature, followed by extraction with ethyl acetate. Nearly quantitative yields were obtained in a very short time, and the derivatives showed high sensitivity in the electron capture detector (ECD). We now report the logical extension of this work to the analysis of phenolic acids.

EXPERIMENTAL

Reagents and chemicals

o-Hydroxyphenylacetic acid (*o*-HPAA), *p*-hydroxyphenylacetic acid (*p*-HPAA), *p*-hydroxyphenyllactic acid (*p*-HPLA) and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from Sigma (St. Louis, Mo., U.S.A.), *m*-hydroxyphenylacetic acid (*m*-HPAA), vanillic acid (VA), homovanillic acid (HVA) and vanilmandelic acid (VMA) from Tokyo Kasei Kogyo (Tokyo, Japan) and *p*-hydroxymandelic acid (*p*-HMA) from Nakarai (Kyoto, Japan). VA was recrystallized from water. Pentafluorobenzoyl chloride was purchased from Pierce (Rockford, Ill., U.S.A.). Dinitrophenyl (DNP)-phenylalanine, purchased from Wako (Osaka, Japan), and 2,4,6-trichlorophenyl 4′-nitrophenyl ether (MO), a generous gift from Mitsui Toatsu Kagaku (Tokyo, Japan), were utilized as internal standards. Diethyl ether was treated with an acidic iron(II) sulphate solution to remove peroxides, according to the procedure previously described[2]. All the other chemicals used were of reagent grade purity.

Procedure

Derivatization. An aqueous aliquot containing from 1 to 5 μg of each phenolic acid (5-HIAA, 2–10 μg) and 1 ml of 5% sodium hydrogencarbonate were pipetted into a 10-ml glass-stoppered test-tube. The total volume was made up to 2 ml with

water and 0.04 ml of pentafluorobenzoyl chloride were added to this solution. The solution was then shaken at 300 rpm for 10 min at room temperature. The reaction mixture was acidified to pH 1 with 10% H_3PO_4 (0.5 ml), saturated with NaCl and 0.5 ml of the methanolic internal standard solution (DNP-phenylalanine: 100 μg/ml; MO: 20 μg/ml) were added. The solution was extracted three times with 3 ml of diethyl ether with vigorous shaking by hand. To the combined ethereal extracts was added 1 ml of methanol, and methylation was carried out by bubbling diazomethane, generated according to the micro-scale procedure of Schlenk and Gellerman[3], through this solution until the yellow colour persisted. After standing for 5 min, the solvents were evaporated to dryness at 50° under a stream of nitrogen. The residue was dissolved in 1 ml of ethyl acetate and the solution was dried over anhydrous Na_2SO_4. This solution was diluted 20 times with *n*-hexane, and 1–2 μl of the resulting solution was injected into the gas chromatograph.

Extraction of phenolic acids from urine. A 0.5 ml portion of urine was adjusted to pH 1 with 6 *N* HCl and saturated with NaCl. The solution was extracted five times with 2 ml of diethyl ether by mechanical shaking for 5 min. The interface emulsion was broken by centrifugation for 3 min at 400 *g*. The combined ethereal extracts were dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness under reduced pressure. The residue was dissolved in 1 ml of 5% sodium hydrogencarbonate and the derivatization was then carried out.

Gas chromatography. A Shimadzu Model 3BE gas chromatograph equipped with a ^{63}Ni ECD, operated in the pulse mode, and a glass column (1.7 m × 3 mm I.D.) packed with 5% OV-17 on Gas-Chrom P (80–100 mesh) silanized was utilized. The column packing was prepared by the "solution coating" technique[4]. The column was conditioned with a nitrogen flow-rate of *ca.* 20 ml/min at 280° for 20 h. Gas chromatography was performed at the column temperatures indicated in the figures. The nitrogen flow-rate was 40 ml/min.

RESULTS AND DISCUSSION

Two-step reactions were employed for the preparation of O-pentafluorobenzoyl methyl ester (PFB–Me) derivatives of phenolic acids. In the first step, both the alcoholic and phenolic hydroxyl groups of phenolic acids were pentafluorobenzoylated with pentafluorobenzoyl chloride in an aqueous alkaline medium. In the second step, the resulting derivatives, which were extracted with diethyl ether in an acidic medium, were methylated with diazomethane. In the pentafluorobenzoylation reaction the formation of small amounts of the mixed anhydrides of O-pentafluorobenzoylated phenolic acids and pentafluorobenzoic acid was observed. These anhydrides, however, could be extracted with diethyl ether, and they were easily converted into the PFB–Me derivatives by contacting them with methanol during methylation. No precaution to exclude moisture is necessary for these reactions and, therefore, the preparation of derivatives could be carried out rapidly and conveniently.

Chromatograms of representative phenolic acids are shown in Figs. 1 and 2. Each acid gave a single and symmetrical peak. The structure of the derivatives prepared by the procedure described above was elucidated by gas chromatography–mass spectrometry (GC–MS). A molecular ion peak (M^+) which is consistent with the structure postulated was observed for each of the derivatives, and the other common

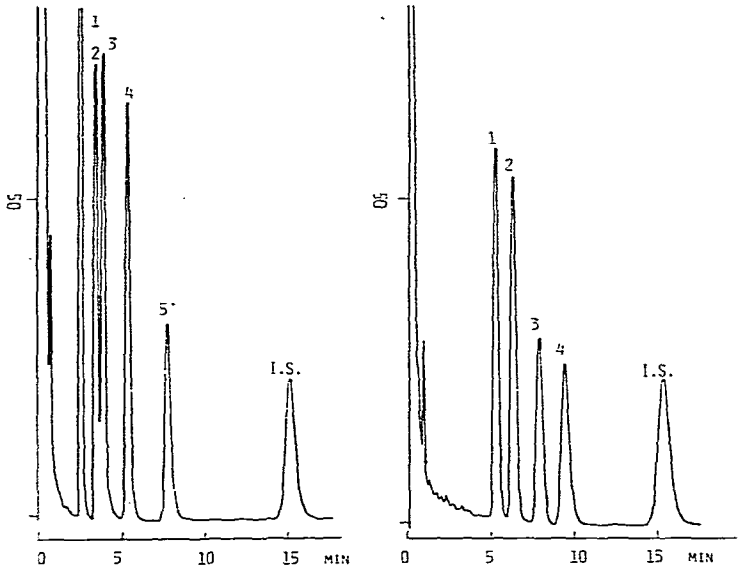

Fig. 1. Chromatogram of the PFB–Me derivatives obtained from a phenolic acid standard mixture. Each peak represents 125 pg of phenolic acid. I.S. = 250 pg of 2,4,6-trichlorophenyl 4′-nitrophenyl ether (MO). Column and detector temperature, 210°. Peaks: 1 = *o*-hydroxyphenylacetic acid; 2 = *m*-hydroxyphenylacetic acid; 3 = *p*-hydroxyphenylacetic acid; 4 = vanillic acid; 5 = homovanillic acid.

Fig. 2. Chromatogram of the PFB–Me derivatives obtained from a phenolic acid standard mixture. Each peak represents 250 pg of phenolic acid. I.S. = 1.25 ng of DNP-phenylalanine methyl ester. Column and detector temperature, 260°. Peaks: 1 = *p*-hydroxymandelic acid; 2 = *p*-hydroxyphenyllactic acid; 3 = vanilmandelic acid; 4 = 5-hydroxyindole-3-acetic acid.

fragment-ion peaks which were useful for structure elucidation were $M^+ - 32$ (CH_3OH), $M^+ - 59$ ($COOCH_3$) and *m/e* 195 (C_6F_5CO). For the derivative of 5-HIAA, a peak with *m/e* 340 ($M^+ - 59$) was the base peak, and for the other derivatives a peak with *m/e* 195 was the base peak in each case. The results of GC–MS demonstrated that both the alcoholic and phenolic hydroxyl groups are pentafluorobenzoylated and that the indole ring nitrogen of 5-HIAA is not.

These PFB–Me derivatives were very stable under normal laboratory conditions. A similar stability was observed for the derivatives of HVA, VMA and 5-HIAA obtained from urinary extracts.

The electron-capture responses of the derivatives are extremely high and the lowest amount of each acid to give a peak twice as high as the noise under our instrumental conditions can be summarized as follows: *o*-HPAA, *m*-HPAA, *p*-HPAA and VA, 1 pg; HVA, 2 pg; *p*-HMA and *p*-HPLA, 2.5 pg; VMA, 5 pg; 5-HIAA, 10 pg.

In order to test the linearity of the calibration graphs, various amounts of the phenolic acid ranging from 1 to 5 μg (5-HIAA, 2–10 μg) were derivatized in a mixture and aliquots representing 50–250 pg (5-HIAA, 100–500 pg) of the acids were injected into the gas chromatograph. In each case, the response of the ECD, as measured as the peak height ratio relative to the internal standard, was linear in the range studied and its reproducibility was found to be satisfactory.

Attempts were made to apply the present method to the simultaneous determination of urinary HVA, VMA and 5-HIAA, which are of diagnostic importance in various diseases. Interfering peaks, with MO and DNP-phenylalanine methyl ester as the internal standards, were not observed in several urine samples investigated. Normal urine samples were fortified with various known amounts of HVA, VMA and 5-HIAA (each 1–5 μg per 0.5 ml of urine) and the average recoveries for the entire procedure were determined as follows: HVA, 103%; VMA, 89%; 5-HIAA, 90%. Chromatograms obtained from normal adult urine and the same urine fortified with these phenolic acids are shown in Figs. 3 and 4. The ethereal extracts obtained from 10 ml of a normal urine sample were derivatized for the GC–MS analysis, and the mass spectra obtained from the peaks of HVA, VMA and 5-HIAA derivatives were identified by comparison with those obtained from the authentic standards. It was shown that no impurity is present in each peak.

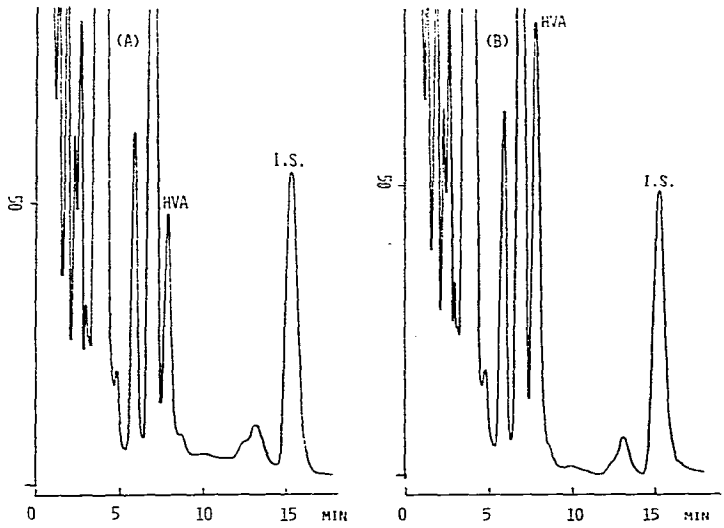

Fig. 3. Chromatograms obtained from 0.5 ml of normal adult urine (A) and the same urine fortified with 2.5 μg of homovanillic acid (HVA) (B). I.S. = 2,4,6-Trichlorophenyl 4'-nitrophenyl ether (MO). Column and detector temperature 210°.

There have been a number of reports dealing with the determination of HVA and/or VMA[5–10] and 5-HIAA[6,11] in biological fluids using GC–ECD. However, to our knowledge, a method for the simultaneous determination of these phenolic acids in urine using GC–ECD has not hitherto been reported. A significant advantage of the present method is that the PFB–Me derivatives are stable to moisture and, therefore, reliable quantitation can be carried out.

The presence of abnormal amounts of HVA, VMA and 5-HIAA is a useful indicator of diseases associated with the metabolism of biological amines such as epinephrine, norepinephrine and serotonin[12,13]. It is anticipated that this convenient method will find extensive applications for the determination of these acids in routine and research analyses.

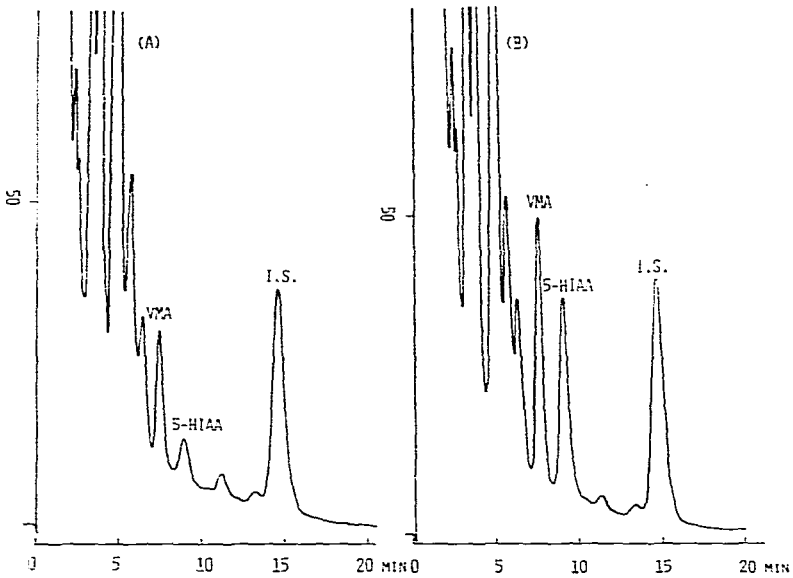

Fig. 4. Chromatograms obtained from 0.5 ml of normal adult urine (A) and the same urine fortified with 2.5 μg of vanilmandelic acid (VMA) and 5 μg of 5-hydroxyindole-3-acetic acid (5-HIAA) (B). I.S. = DNP-phenylalanine methyl ester. Column and detector temperature, 260°.

ACKNOWLEDGEMENT

This work was supported in part by a grant for scientific research from the Ministry of Education, Science and Culture, which is gratefully acknowledged.

REFERENCES

1 M. Makita, S. Yamamoto, K. Ono and T. Katagi, *95th Annual Meeting of the Pharmaceutical Society of Japan, Nishinomiya, Hyogo, Japan, April 1975.*
2 M. Makita, S. Yamamoto and M. Kono, *J. Chromatogr.*, 120 (1976) 129.
3 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
4 E. C. Horning, W. J. A. VandenHeuvel and B. G. Greek, *Methods Biochem. Anal.*, 11 (1963) 69.
5 S. W. Dziedzic, L. M. Bertani, D. D. Clarke and S. E. Gitlow, *Anal. Biochem.*, 47 (1972) 592.
6 E. Watson, S. Wilk and J. Roboz, *Anal. Biochem.*, 59 (1974) 411.
7 S. Yoshiue, M. Yoshioka and Z. Tamura, *Chem. Pharm. Bull.*, 23 (1975) 3040.
8 S. W. Dziedzic, L. B. Dziedzic and S. E. Gitlow, *J. Lab. Clin. Med.*, 82 (1973) 829.
9 S. W. Dziedzic and S. E. Gitlow, *J. Neurochem.*, 22 (1974) 333.
10 B. Sjöquist and E. Änggård, *Anal. Chem.*, 44 (1972) 2297.
11 B. L. Goodwin, C. R. J. Ruthven, M. W. Weg and M. Sandler, *Clin. Chim. Acta*, 62 (1975) 439.
12 J. Nordmann and R. Nordmann, *Advan. Clin. Chem.*, 4 (1961) 53.
13 C. M. Williams and C. C. Sweeley, in H. A. Szymanski (Editor), *Biomedical Applications of Gas Chromatography*, Vol. 1, Plenum, New York, 1964, p. 225.