Journal of Chromatography, 210 (1981) 342–345
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,695

Note

Gas chromatographic enzymic determination of amygdalin

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(Received January 21st, 1981)

The evaluation of amygdalin (D-mandelonitrile-β-D-gentiobioside) as a cancer remedy has been the subject of considerable controversy[1,2]. Recently, Stobaugh *et al.*[3] developed a method for the determination of amygdalin in human plasma, based on the gas chromatographic (GC) determination of benzaldehyde formed by enzymatic (β-glucosidase) hydrolysis of the parent glycoside. In this paper, we describe a more sensitive method for the determination of amygdalin in aqueous solution. Enzymatic hydrolysis of amygdalin by β-glucosidase was performed according to the procedure described by Stobaugh *et al.*[3], then the benzaldehyde liberated was converted by pentafluorobenzyloxylamine into its O-pentafluorobenzyl oxime and the derivative was determined by GC with the use of a flame-ionization detector (FID) or an electron-capture detector (ECD).

EXPERIMENTAL

Reagents

Pentafluorobenzyloxylammonium [O-(2,3,4,5,6-pentafluorobenzyl)hydroxylammonium] chloride (PFBOA) was synthesized[4] from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan). α,α-Dichlorodiphenylmethane was used as an internal standard (IS). Amygdalin (Merck, Darmstadt, G.F.R.) was used without further purification (m.p. 221–222°C). β-Glucosidase (powder, prepared from almonds) was obtained from Sigma (St. Louis, MO, U.S.A.), with an activity of 4.0 units/mg (1 unit will liberate 1.0 μmole/min of glucose from Salicin at pH 5.0 at 37°C). Benzaldehyde was distilled and kept under nitrogen.

Apparatus and conditions

A Shimadzu GC-4APF gas chromatograph equipped with a FID and GC-4APE gas chromatograph equipped with a 10-mCi ^{63}Ni ECD were used. A 2-m glass column packed with 3% XE-60 on 80–100-mesh Celite 545 (AW DMCS) was used, with a column temperature of 160°C, a detector temperature of 180°C and a chart speed of 0.25 cm/min.

0021-9673/81/0000–0000/$02.50

Standard procedure

Method A. When the FID was used, amygdalin was dissolved in 50 m*M* acetate buffer (pH 4.8) to give solutions of concentration 2.5–10 μg/ml. To 0.2 ml of the sample solution in a 5 ml centrifuge tube were added 50 μl of β-glucosidase (2.0 units/ml) dissolved in acetate buffer (50 m*M*, pH 4.80). After shaking for 1 h in an incubator of 30°C, 50 μl of an aqueous solution of PFBOA (2.2 mg/ml) were added and the mixture was allowed to stand for 1 h at room temperature. After being saturated with sodium chloride and acidified with 1 drop of 18 *N* sulphuric acid, the O-PFBO derivative of benzaldehyde was extracted with 500 μl of ethyl acetate containing 1 μg of α,α-dichlorodiphenylmethane as an IS, followed by centrifuging for a few minutes to separate two phases. The lower, aqueous layer was removed with a syringe with a long needle, a few grains of anhydrous sodium sulphate were added to dry the ethyl acetate extract, followed by concentration of the solvent into a small volume under a stream of air, and an aliquot of the final solution was injected on to the GC column.

Method B. When the ECD was used, amygdalin was dissolved in 50 m*M* acetate buffer (pH 4.8) to give solutions of concentration 0.5–2.5 μg/ml. Then 0.2 ml of the sample solution was treated by a technique similar to method A, except that this time 2.0 ml of ethyl acetate containing 5 μg of α,α-dichlorodiphenylmethane was used for extraction of the O-PFBO derivative.

RESULTS AND DISCUSSION

Stobaugh *et al.*[3] hydrolysed 3-ml plasma samples spiked with amygdalin at concentrations of 2–20 μg/ml. If a molar equivalent of benzaldehyde was produced by the hydrolytic action of β-glucosidase (in their work, the benzaldehyde recovered was found to be 95% of the amygdalin added), about 1.4 μg of benzaldehyde was produced from 6 μg of amygdalin. They extracted it with 250 μl of chloroform containing an IS and injected directly 1 μl of it on to a gas chromatograph equipped with an FID. However, when GC with an FID was used, the sensitivity limit is about 2 μg in 250 μl of benzaldehyde in ethyl acetate, partly because benzaldehyde is so volatile that it is difficult to concentrate a solution without loss, and partly because the peak of benzaldehyde on the chromatogram occurs on the slope of that of solvent. Hence, Stobaugh *et al.* were forced to take 3-ml plasma samples and to extract with as small a volume as 250 μl of organic solvent.

We have found PFBOA to be an excellent derivatizing agent in the GC determination of low-molecular-weight carbonyl compounds in aqueous solution. The reaction of carbonyl compounds with PFBOA proceeded readily in weakly acidic media (pH 2–5) at room temperature to yield derivatives extractable from the aqueous solution with organic solvents, and the complete removal of the unreacted reagent was easily achieved. The resulting derivatives were stable in organic solvents and very volatile, and therefore the GC separation could be carried out at low temperatures. Also, the O-PFBO were extremely sensitive to the ECD. In this work, PFBOA was applied to the micro-determination of amygdalin. The reagent concentration was made 100–1000-fold greater than that of benzaldehyde to ensure completion of the condensation reaction with a small amount of the aldehyde in the aqueous solution. The conditions for enzymatic hydrolysis of amygdalin were fixed according to the procedure of Stobaugh *et al.*

Experiments were also conducted to determine the effect of pH on β-glucosidase activity. The enzyme rapidly hydrolysed amygdalin to liberate benzaldehyde between pH 4.0 and 7.0. Therefore, incubation solutions were buffered at pH 4.8, taking into consideration conditions for the enzyme activity of β-glucosidase and the condensation reaction with PFBOA. Ethyl acetate was a suitable solvent for the extraction of the oxime. Salting-out improved the extent of extraction. In order to prevent an excess of PFBOA from being extracted, extraction was carried out in the acidic media by adding 1 drop of 18 *N* sulphuric acid.

Typical GC separations are illustrated in Fig. 1. α,α-Dichlorodiphenylmethane was used as an internal standard. Quantitative assay was carried out by comparison of the peak-height ratio with a calibration graph prepared from solutions containing known amounts of amygdalin.

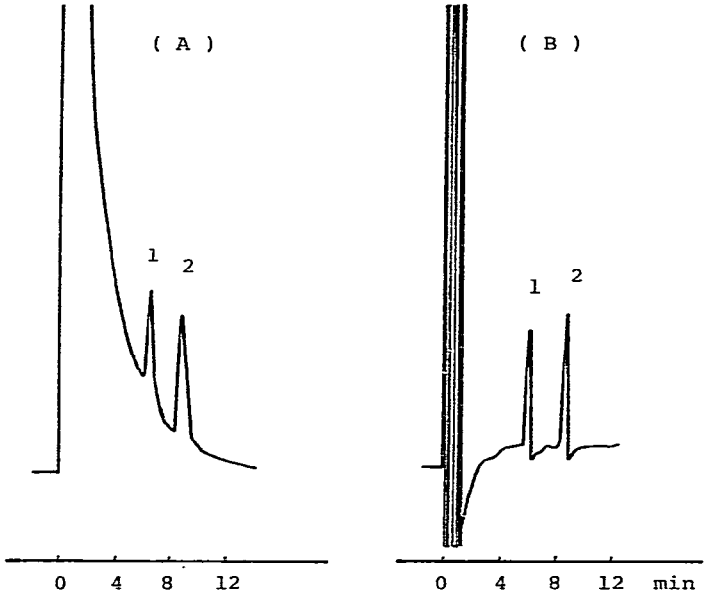

Fig. 1. Gas chromatogram of benzaldehyde produced from hydrolysis of amygdalin by β-glucosidase on a 2.0-m 3% GE-XE column at 160°C. 1 = Benzaldehyde O-pentafluorobenzyloxime; 2 = α,α-dichlorodiphenylmethane. (A) Analysis on a gas chromatograph equipped with a hydrogen FID, using 2.0 μg of amygdalin. (B) Analysis on a gas chromatograph equipped with a ^{63}Ni ECD, using 200 ng of amygdalin.

In the present method, the reaction of benzaldehyde with PFBOA provided a larger molecule, which led to an increase in FID sensitivity and to the possibility of concentration of the extract without perceptible loss of the derivative. Even when an FID was used, the sensitivity limit was improved 10-fold compared with the procedure performed without derivatization by Stobaugh *et al.*, which means that one tenth of the amount of plasma samples is sufficient for analysis. The O-PFBO derivative was very sensitive towards the ECD and was about 40 times more sensitive than α,α-dichlorodiphenylmethane used as an IS. Fig. 1B is a chromatogram obtained on treating 200 ng of amygdalin according to method B. A stable response was observed, even for the injection of 10 pg of benzaldehyde as its O-PFBO.

The overall precision of measurements by method B was a coefficient of variation of 5.03% ($n = 6$) and a correlation coefficient of 0.9989 over the range 0.1–2.0 μg of amygdalin. The advantage of the proposed method is the potential for microdetermination with fairly good precision. The procedure will be useful for monitoring the concentration and behaviour of amygdalin in biological materials.

ACKNOWLEDGEMENTS

We thank Dr. S. Kawachi, Kawachi Clinic, for supplying amygdalin, Mr. M. Watanabe, Japan Macrobiological Medical Association, for his lucid discussions of amygdalin and Miss T. Imaeda, student, for assistance with the experiments.

REFERENCES

1 B. J. Culliton, *Science*, 182 (1973) 1000.
2 M. Zucker, *Let's Live*, 65 (1979) 65.
3 J. F. Stobaugh, L. A. Sternson and A. J. Repta, *Anal. Lett.*, 9 (1978) 753.
4 K. Kobayashi, M. Tanaka and S. Kawai, *J. Chromatogr.*, 187 (1980) 413.