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Note

Separation of isomeric (hydroxyphenyl)acetic acids by reversed-phase high-performance liquid chromatography

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The separation of *ortho*, *meta*, and *para* isomers by high-performance liquid chromatography (HPLC) is desirable for resaons of speed and quantitation. The problems encountered with paper and thin-layer chromatography, such as lack of precise quantitation, or with gas–liquid chromatography (GLC), which often requires derivatization and sometimes leads to thermal degradation, are not common to HPLC[1].

Wulf and Nagel[1] have demonstrated the separation of *o*- and *p*-hydroxybenzoic acid isomers on μBondapak C_{18}®*. Waters Assoc. (Milford, Mass., U.S.A.) has also indicated the separation of *o*-, *m*-, and *p*-benzenedicarboxylic acid isomers on μBondapak C_{18}.

Excessive excretion of (*p*-hydroxyphenyl)acetic acid occurs in disorders such as cystic fibrosis, scurvy, steatorrhoea, macrocytic anemia, and tyrosinosis of the newborn[2]. Improved methods of analysis for this metabolite could be of great importance. Methods for the determination of (*o*-hydroxyphenyl)acetic acid in disorders of phenylalaninemia[3] and phenylketonuria[4] are also important. In our research, a separation of *o*-, *m*-, and *p*-isomers of (hydroxyphenyl)acetic acid was needed for some plant isolation work. It is the purpose of this note to report a facile separation of these three isomers.

MATERIALS AND METHODS

Apparatus

A Waters Assoc. ALC-100 liquid chromatograph equipped with an M-6000 pump, a U6K injector, and a Model 440 absorbance detector (12.5 μl volume) was

* Mention of a trade name or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

used for all separations. A Waters Assoc. μBondapak C_{18} column (30 × 0.4 cm I.D.), particle size 10 μm, was selected for the separations.

Reagents

Phenylacetic acid and *o*-, *m*-, and *p*-isomers of (hydroxyphenyl)acetic acid were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Distilled water, methanol distilled-in-glass from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.), and analyzed reagent acetic acid from J. T. Baker (Phillipsburg, N.J., U.S.A.) were used as eluents for the liquid chromatography.

Column chromatographic procedure

Solutions of (*o*-hydroxyphenyl)acetic acid (1.0%), (*m*-hydroxyphenyl)acetic acid (0.85%), (*p*-hydroxyphenyl)acetic acid (1.0%) and phenylacetic acid (4%) in methanol were prepared for injection onto the μBondapak C_{18} column (volumes of solutions are indicated in the figures). Detection was by ultraviolet absorption at 254 nm.

First, the retention volume (R_v) of phenylacetic acid was determined with the following eluents: methanol, methanol–water (9:1), methanol–water (3:1), methanol–water (1:1), methanol–5% acetic acid (1:1), and methanol–5% acetic acid (1:3). Then the retention volumes of each of the three (hydroxyphenyl)acetic acids were determined individually with methanol–5% acetic acid (1:3) as the eluent. A mixture of these three isomers and phenylacetic acid then was injected with the same eluent to visualize the separation.

Finally, a mixture of the three (hydroxyphenyl)acetic acids was injected with the following eluents: methanol–5% acetic acid (3:17), methanol–5% acetic acid (1:9), and 5% acetic acid.

RESULTS AND DISCUSSION

The R_v value of phenylacetic acid was found to vary little with changes in the eluent concentration from straight methanol to methanol–water (1:1). The polarity of the molecule due to its ionization caused it to elute near the void volume. In order to increase the R_v of phenylacetic acid, one must suppress the ionization of the molecule to decrease its polarity by the addition of acetic acid to the eluent. The use of methanol–5% acetic acid (1:3) gave a satisfactory R_v for phenylacetic acid and served as a starting point for the resolution of the three (hydroxyphenyl)acetic acids.

The optimal separation of *o*-, *m*-, and *p*-isomers of (hydroxyphenyl)acetic acid with reference to phenylacetic acid is illustrated in Fig. 1. The small unidentified peak following the *ortho* isomer is an impurity in the *meta* isomer. The separation shown in Fig. 1 occurred because of the differences in reduced polarity of the isomers in the presence of acetic acid.

The effect of reducing the methanol concentration on the elution of the isomeric (hydroxyphenyl)acetic acids is shown in Fig. 2. The retention volumes of the isomers increase as the concentration of methanol in the eluent decreases. The separation between the *meta* and *ortho* isomers almost disappears; however, the separation

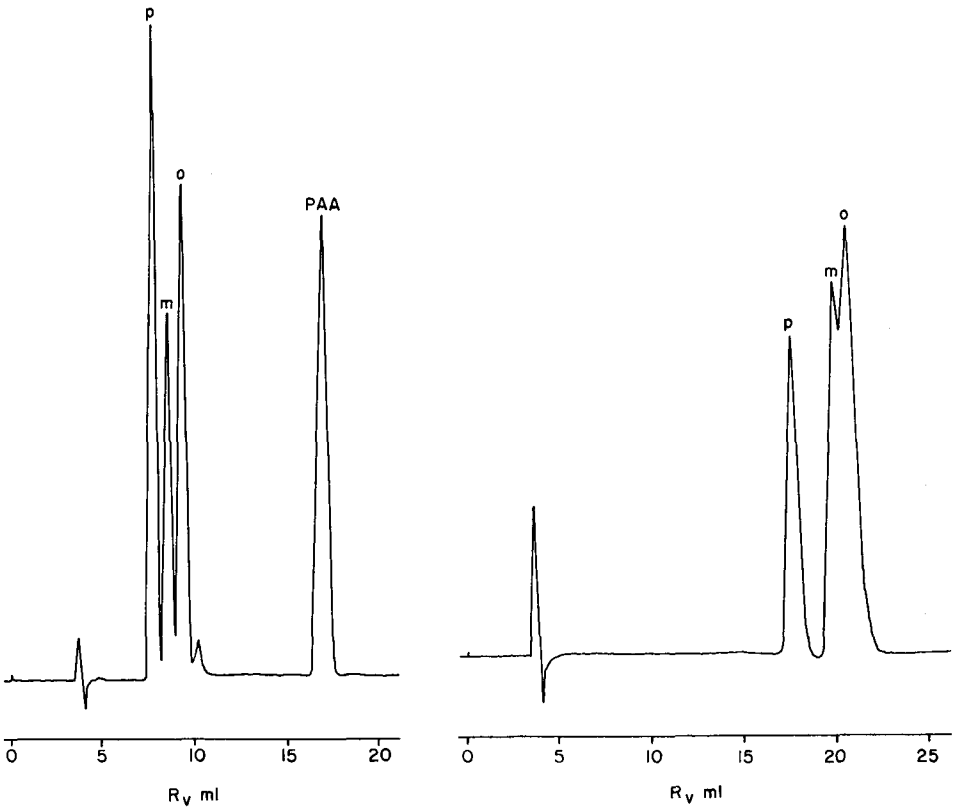

Fig. 1. Optimal separation of *o*-, *m*-, and *p*-isomers of (hydroxyphenyl)acetic acid with reference to phenylacetic acid (PAA). Column, μBondapak C_{18} (30 × 0.4 cm. I.D.), particle size, 10 μm. Eluent, methanol–5% acetic acid (1:3). Detection, 254 nm; attenuation, 0.5 absorbance units. Sample size, 20 μl (5 μl of each component solution). Flow-rate, 1 ml/min. Temperature, ambient.

Fig. 2. Effect of reducing the methanol concentration on the elution of (hydroxyphenyl)acetic acids. Same conditions as in Fig. 1 except: eluent, 5% acetic acid; sample size, 25 μl (5 μl of *o*-isomer solution and 10 μl of each of the *m*- and *p*-isomer solutions).

of the *para* and *meta* isomers is maintained. The differences in retention volumes again are attributable to differences in reduced polarity of the isomers in the presence of acetic acid.

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