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SALTING-OUT CHROMATOGRAPHIC SEPARATION OF HYDROXYBENZOIC ACID ISOMERS

WATARU FUNASAKA, KAZUMI FUJIMURA AND SUE KUSHIDA

Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo-ku, Kyoto (Japan)

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SUMMARY

The separation of hydroxybenzoic acid isomers by salting-out chromatography using Amberlite CG-50 (Na^+ form) as stationary phase and acidic sodium chloride solution as mobile phase has been studied. The distribution coefficients of hydroxybenzoic acids increased with increase in the concentration of sodium chloride and with decrease in the pH of the solution. The best separation was achieved when a mixture of the *o*- and *m*-isomers or the *o*- and *p*-isomers was eluted at 40 ± 1° through a column of length 150 mm and I.D. 10 mm with 2 *M* sodium chloride solution of pH 1.8. The determination of the acids in the eluates by UV spectrophotometry proved that the recovery of the acids was almost quantitative.

INTRODUCTION

Salting-out chromatography, in which an ion-exchange resin is used as stationary phase and an inorganic salt solution as mobile phase, is one of the useful techniques for the separation of homologues[1] or isomers[2,3]. The behaviour of water-soluble organic compounds in salting-out sorption on ion-exchange resins has been attributed to the van der Waals' interaction between the hydrocarbon part of the organic compound and the resin matrix, as well as to the salting-out effect of the inorganic salt.

The separation of hydroxybenzoic acid isomers, which are used as intermediates for dyestuffs, preservatives in foods, etc., is usually achieved by paper chromatography[4–6], thin-layer chromatography[7–9] or gas chromatography after esterification[10,11]. These methods are, however, rather unsatisfactory in terms of quantitative recovery. The present paper deals with the application of salting-out chromatography to the quantitative separation of hydroxybenzoic acid isomers, as well as with their determination by UV spectrophotometry.

EXPERIMENTAL

Materials

Sample. Three isomers of hydroxybenzoic acid (reagent grade; Tokyo Kasei Kogyo Co. Ltd.) were purified by recrystallization from distilled water until the

melting points agreed with the literature values. Standard solutions, 200 μg/ml or 100 μg/ml, were prepared by dissolving the purified compounds in distilled water. Solutions of a mixture of two isomers, containing 100 μg of each isomer in 1 ml, were prepared from each standard solution.

Resin. A weakly acidic cation-exchange resin, Amberlite CG-50, was used. After conditioning in the usual way, the resin was converted to the Na^+ form with sodium chloride solution and was air-dried at room temperature by spreading on a filter paper. The resin of 100–200 mesh was used for batch operation and that of 200–400 mesh for column operation.

Apparatus

A spectrophotometer (Shimadzu QV-50) was used for the spectrophotometric determination of hydroxybenzoic acids and a pH meter (Toa Denpa HM-5A) for measurement of the pH of the solution. From the column effluents, 5-ml portions were collected successively by using a fraction collector (Toyo SF-200A).

Methods

Measurement of distribution coefficients. The distribution coefficients of hydroxybenzoic acid isomers on the resin (Na^+ form) and their dependence on the concentration of the salting-out reagent and the pH of the solution were measured by batch operation at room temperature. To 1 g of the resin, which had been weighed into a 50-ml conical flask fitted with a glass stopper, were added 25 ml of an aqueous sodium chloride solution of various concentrations and pH values adjusted with 6 *N* hydrochloric acid. The flask was shaken gently for 1 h in order to swell the resin, and then 1 ml of hydroxybenzoic acid solution, containing 100 μg of hydroxybenzoic acid, was added to the flask. After being shaken vigorously for 1 h on a mechanical shaker, the flask was allowed to stand for 24 h for equilibration and then the resin was separated from the aqueous phase by filtration by using a sintered-glass filter of No. 2 size without suction. The amount of hydroxybenzoic acid remaining in the solution was determined spectrophotometrically and the distribution coefficient (D) was calculated in the usual way. The pH values of each equilibrated solution were also recorded.

Column preparation. An appropriate volume of the sodium chloride solution to be used as eluent was poured on to the 200–400-mesh resin, which had been placed in a large beaker, and then the resin was stirred gently with a magnetic stirrer. After swelling of the resin, hydrochloric acid (6 *N*) was added dropwise with a pipette and the pH of the solution was adjusted to the required value by using a pH meter. After complete equilibration between the resin and the solution had been attained, the resin was separated from the solution by decantation and poured into a glass chromatographic tube of dimensions 150 × 10 mm or 230 × 12 mm, fitted with a jacket and a stopcock. The equilibrated solution obtained by the above procedure was used as eluent*.

* A carboxylic acid type cation-exchange resin, *e.g.*, Amberlite CG-50, is expected to exert a buffer action in acidic region, when it is used in the salt form, such as the Na^+ form. It would be difficult and time-consuming, therefore, to pack the column with the resin in the Na^+ form that has been swollen in water alone and to equilibrate the resin by washing the column with an acidic sodium chloride solution prepared independently.

Chromatography. As described in a previous paper[12], the rate of salting-out adsorption of organic acids is generally slow at room temperature because of the slow diffusion of the solute to the resin phase in a concentrated salting-out reagent solution. In the present work the chromatography was performed at 40° by circulating water, maintained at 40 ± 1° by a thermostat, through a column jacket with a pump.

One hour after the circulation of water was started, 1 ml of working mixture was added to the top of the column with a pipette, and then the elution was started.

Analysis. Hydroxybenzoic acids were determined by UV spectrophotometry using a quartz cell of 1.00 cm path length. In order to establish the most suitable wavelength for determination, absorption curves were first measured. Aqueous sodium chloride solutions of various concentrations and pH were used as solvent and reference. As shown in Fig. 1, the absorbance of hydroxybenzoic acids was strongly affected by the pH of the solvent, but not by the concentration, and so the measurements were made at the isobestic points, *i.e.*, at 230.0 nm for the *o*-isomer, 223.0 nm for the *m*-isomer and 247.5 nm for the *p*-isomer.

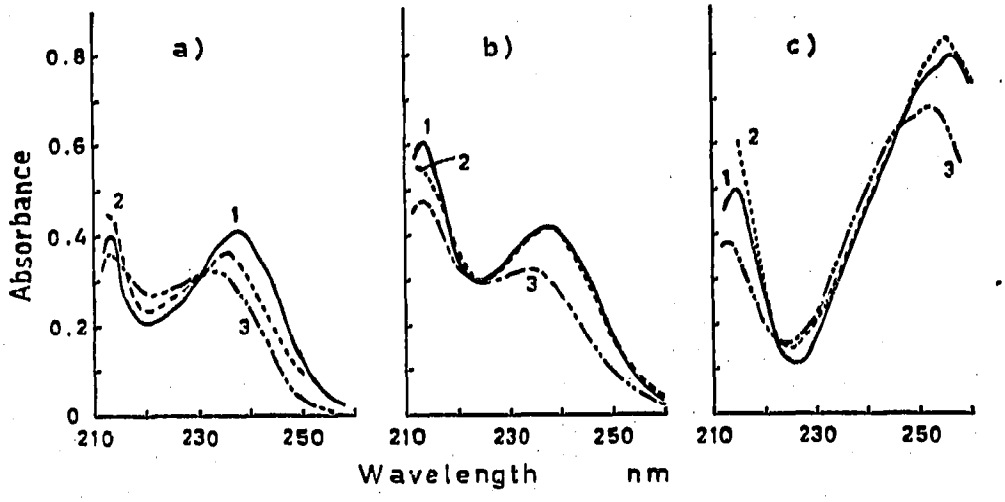


Fig. 1. Effect of the pH of the solvent on the absorption spectra of hydroxybenzoic acids. (a) *o*-isomer, 7.69 μg/ml, at pH: (1) 1.6, (2) 2.4, (3) 3.6; (b) *m*-isomer, 7.69 μg/ml, at pH: (1) 1.3, (2) 2.1, (3) 3.7; (c) *p*-isomer, 7.69 μg/ml, at pH: (1) 1.4, (2) 2.2, (3) 4.0.

RESULTS

Effects of salting-out reagent and pH on the distribution coefficient

The general effects of the concentration and the pH of the salting-out reagent solution on the distribution coefficient can be seen from Fig. 2.

It follows from Fig. 2 that the distribution coefficient, D, increases with increase in the concentration of sodium chloride solution if the pH of the solution is constant, and that it increases with decrease in the pH.

The separation factors between the *o*- and *m*-isomers and between the *o*- and *p*-isomers were 2.5–2.2 and 4.0–3.0, respectively, when 4 *M* sodium chloride solution of pH 1.5–3.0 was used as eluent, and were 2.4–1.6 and 2.0, respectively, when 2 *M* sodium chloride solution of pH 1.5–2.5 was used as eluent. This suggests that the *o*- and *m*-isomers and also the *o*- and *p*-isomers may be easily separated under these conditions.

As there is little or no difference between the distribution coefficients of the *m*- and *p*-isomers, the simultaneous separation of the three isomers was not possible.

The addition of ethanol to the salting-out reagent solution was found to cause a decrease in the distribution coefficients, as shown in Fig. 3.

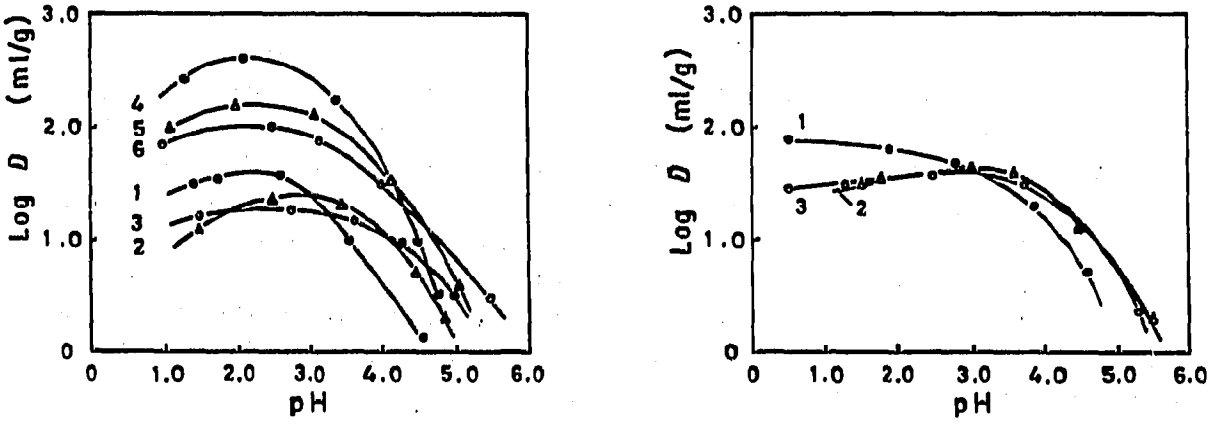


Fig. 2. Effects of the concentration and the pH of the sodium chloride solution on the distribution coefficients of hydroxybenzoic acids. (1) *o*-isomer in 2 *M* NaCl; (2) *m*-isomer in 2 *M* NaCl; (3) *p*-isomer in 2 *M* NaCl; (4) *o*-isomer in 4 *M* NaCl; (5) *m*-isomer in 4 *M* NaCl; (6) *p*-isomer in 4 *M* NaCl.

Fig. 3. Distribution coefficients of hydroxybenzoic acids in 4 *M* sodium chloride solution containing 5% of ethanol. (1) *o*-isomer; (2) *m*-isomer; (3) *p*-isomer.

Separation of the o- and m-isomers

Developments were made on a column of length 150 mm and I.D. 10 mm by adding a mixture containing 100 μg of each of the *o*- and *m*-isomers and then eluting with 4 *M* sodium chloride solution of pH 2.8 at a flow-rate of 0.4 ml/min. Developments were made similarly on a column of the same size by using 2 *M* sodium chloride solution of pH 1.8 at a flow-rate of 0.2 ml/min. The chromatogram thus obtained is shown in Fig. 4, and the recoveries of each isomer are summarized in Table I.

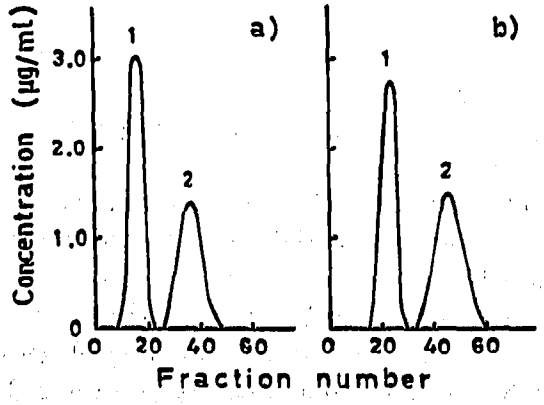


Fig. 4. Separation of the *o*- and *m*-isomers of hydroxybenzoic acid (1) *m*-isomer; (2) *o*-isomer. (a) Column size, 150 × 10 mm; eluent, 2 *M* NaCl of pH 1.8; flow-rate, 0.2 ml/min; column temperature, 40 ± 1°. (b) Column size, 150 × 10 mm; eluent, 4 *M* NaCl of pH 2.8; flow-rate, 0.4 ml/min; column temperature, 40 ± 1°.

TABLE I

RECOVERIES OF HYDROXYBENZOIC ACID ISOMERS

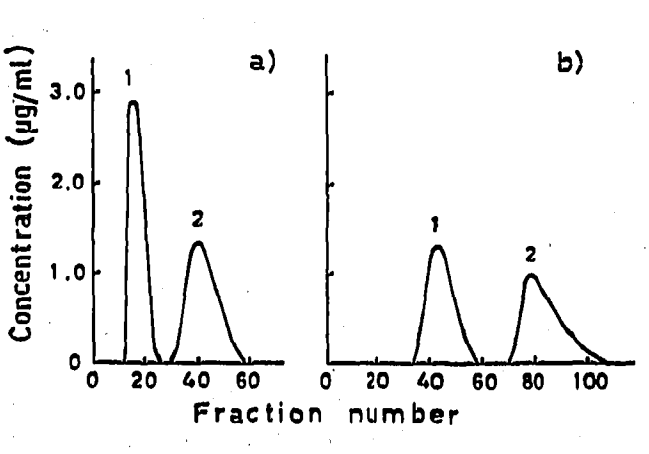
Eluent	*Column*		*Flow-rate* (*ml/min*)	*Sample loaded* (*μg*)			*Recovery* (%)		
	Length (*mm*)	*I.D.* (*mm*)		*o-Isomer*	*m-Isomer*	*p-Isomer*	*o-Isomer*	*m-Isomer*	*p-Isomer*
	150	10	0.4	100	100		101.4	100.4	
	150	10	0.4	100	100		101.1	99.12	
4 *M* NaCl, pH 2.8	230	12	0.4	100		100	98.52		100.5
	230	12	0.4	100		100	101.8		99.75
	150	10	0.2	100	100		100.4	101.1	
	150	10	0.2	100	100		102.3	103.0	
2 *M* NaCl, pH 1.8	150	10	0.2	100		100	102.1		101.2
	150	10	0.2	100		100	99.76		101.6

Separation of the o- and p-isomers

A solution of a mixture of 100 *μg* of each of the *o*- and *p*-isomers was eluted through a column of length 230 mm and I.D. 12 mm with 4 *M* sodium chloride solution of pH 2.8 at a flow-rate of 0.4 ml/min, to give the chromatogram shown in Fig. 5b. The use of a column of length 150 mm and I.D. 10 mm and 2 *M* sodium chloride solution of pH 1.8 as eluent at a flow-rate of 0.2 ml/min gave the chromatogram shown in Fig. 5a. The recoveries are listed in Table I. The results were satisfactory in both cases.

Adsorption isotherm

In order to study the type of salting-out adsorption, the adsorption isotherm of the hydroxybenzoic acids on the cation-exchange resin in 4 *M* sodium chloride solution of pH 1.7 was measured at room temperature (25°) using various concentrations of hydroxybenzoic acid. The procedure was the same as that described for the measurement of distribution coefficients.



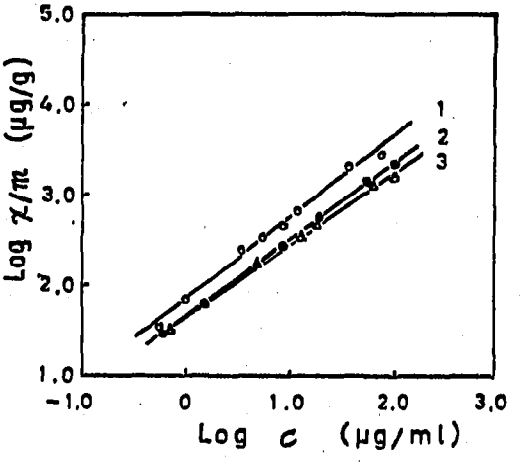


Fig. 5. Separation of the *o*- and *p*-isomers of hydroxybenzoic acid. (1) *p*-isomer; (2) *o*-isomer. (a) Column size, 150 × 10 mm; eluent, 2 *M* NaCl of pH 1.8; flow-rate, 0.2 ml/min; column temperature, 40 ± 1°. (b) Column size, 230 × 12 mm; eluent, 4 *M* NaCl of pH 2.8; flow-rate, 0.4 ml/min; column temperature, 40 ± 1°.

Fig. 6. Adsorption isotherms of hydroxybenzoic acids. (1) *o*-isomer; (2) *m*-isomer; (3) *p*-isomer. Solvent, 4 *M* NaCl of pH 1.7; temperature, 25°.

Fig. 6 shows the plot of the logarithm of the amount adsorbed (x μg) on the resin (m g) against the logarithm of the equilibrium concentration (c μg/ml). A linear relationship clearly exists, and the adsorption isotherm can be expressed by a Freundlich equation, $x/m = a \cdot c^{1/n}$. The constants a and n have the following values: $a = 65.31$ and $n = 1.09$ for the o-isomer; $a = 42.66$ and $n = 1.19$ for the m-isomer; and $a = 43.65$ and $n = 1.24$ for the p-isomer.

DISCUSSION

In salting-out chromatography, inorganic salts of relatively high solubility are generally the most useful as salting-out reagents. In addition, as described earlier[13,14], the higher the valency of the cation the greater is the salting-out action of its inorganic chlorides, if the concentration (normality) is the same. The salting-out effect of divalent metal chlorides on the adsorption of organic acids has been shown[15] to increase in the order: $ZnCl_2 < CaCl_2 < MgCl_2$. This is identical with the order of increasing radii of the hydrated cations.

The distribution coefficients of aromatic carboxylic acids, such as aminobenzoic acid[16] or chlorobenzoic acid[17], are generally too high to allow the use of salts that have a large hydrated ion, and sodium chloride was found to be the most suitable for the separation of hydroxybenzoic acid isomers, in terms of both the absolute and the relative distribution coefficients of the isomers.

The effects of the concentration and the pH of the salting-out solution can be deduced from the variation in the distribution coefficient measured at various concentrations and pH values (Fig. 2). In the process of adsorption, neither the sample nor the resin seems to be dissociated; this is supported by the fact that although the D values are very small when the sample and the resin are both dissociated (pH>5), they increase with the decrease in the pH of the solution. A resin of the weak acid type is, therefore, the most suitable for the non-ionic adsorption of organic carboxylic acids under acidic conditions.

The differences between the distribution coefficients of the isomers can be correlated with the differences in their dissociation constants. In general, the larger the dissociation constant of the acid, the smaller is the adsorptivity[2,16,17]. In the case of hydroxybenzoic acid, however, the adsorptivity of the o-isomer, which has the largest dissociation constant ($K_1 = 1.07 \times 10^{-3}$ at 19°), was larger than that of the m-isomer ($K_1 = 8.7 \times 10^{-5}$ at 19°) or of the p-isomer ($K_1 = 3.3 \times 10^{-5}$ at 19°). This is probably due to the formation of a six-membered ring from the o-isomer by intramolecular hydrogen bonding. In non-ionic adsorption, the adsorptivity of aromatic compounds increases with increase in the number of rings[13] or by ring formation. This is because the greater area over which molecules may come into close contact with the resin results in a greater over-all Van der Waal's attraction.

The adsorptivity is also influenced by the solubility of the sample; a sample of greater solubility is generally less well adsorbed. The finding that the distribution coefficient of the o-isomer is greater than those of the m- and the p-isomers supports this generalization. The decrease in the distribution coefficients in ethanolic salt solution (Fig. 3) can be attributed to the increase in the solubility, which also explains the smaller retention volume of the sample at 40° compared with the value to be expected from Fig. 2.

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