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SEPARATION OF PHENOLIC O-GLUCURONIDES AND PHENOLIC SULPHATE ESTERS BY MULTIPLE LIQUID–LIQUID PARTITION

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SUMMARY

A liquid–liquid partition method is described for the separation of phenolic O-glucuronides from the corresponding phenolic sulphate esters and of the different compounds within the two classes of conjugates. The method, which involves a countercurrent technique with continuous flow of the solvents, is suitable for the isolation of these metabolic conjugates from biological fluids (bile and urine).

INTRODUCTION

One of the steps involved in the metabolic transformation of many foreign compounds and endogenous substances in mammals is their conjugation with glucuronic acid (UDPGA) and/or sulphuric acid (PAPS). Interest in these conjugates because of their metabolic and physiological significance, together with the difficulties encountered in their chemical synthesis, has stimulated attempts to set up preparative methods suitable for their isolation from biological fluids[1–5]. For this purpose, we have studied the application of the liquid–liquid partition (countercurrent distribution) to the separation of phenolic O-glucuronides and phenolic sulphate esters.

The advantages of this method compared with other available methods are its selectivity, the chemical "inertia" and its effectiveness for preparative purposes.

EXPERIMENTAL

Materials

6-Bromo-2-naphthyl-D-glucuronide (acid form) was obtained from Sigma (St. Louis, Mo., U.S.A.), and *p*-nitrophenyl-D-glucuronide (acid form), *p*-nitrophenyl sulphate (potassium salt) and 6-bromo-2-naphthyl sulphate (sodium salt) were purchased from Serva (Heidelberg, G.F.R.).

The solvents used were analytical-grade reagents and all were distilled before use. In particular, *n*-butanol was pre-treated with neutral alumina so as to eliminate UV-absorbing impurities.

Apparatus

Instead of the conventional Craig machine, the countercurrent apparatus developed by Hietala[6] was used. The apparatus including the glassware was manufac-

tured by Karpinnen Oy (Helsinki, Finland) and the mechanical parts by the Laboratory of Technology, Lepetit (Milan, Italy). The apparatus is a continuous flow countercurrent system with one stationary and one moving phase (upper or lower). The phase ratio $\alpha = v_m/v_s$ (v_m and v_s are the volumes of the moving and stationary phases, respectively) is chosen before each fractionation and can be decreased to 0.2–0.3, depending on the solvent system employed.

The apparatus consists of 150 glass units (unit volume = 13.5 ml) connected in series of ten with PTFE tubing. Shaking is accomplished by the rotation of the distribution train axle by ±45°. A DLC peristaltic pump for organic solvents (E.A. Hughes, Epsom, Great Britain) and an LKB (Stockholm, Sweden) Model 7000 Ultrorac fraction collector are used as ancillary equipment.

Selection of the operating conditions

Because the separation is at a maximum in the Martin–Synge distribution[7] when $v_m/v_s = 0$, the lowest phase ratio ought to be selected in order to achieve a high resolution. In our partition studies, the phase ratios were chosen between 0.42 and 0.28. A shaking frequency of 30 cycles per minute, an amplitude of ±45° and a flow-rate of 1–2 ml/min were used in these experiments.

Fractionation

At the beginning of a fractionation, the conjugate mixture was dissolved in the stationary phase and loaded into the first tubes (0, 1, 2 and 3) of the apparatus, keeping the phase ratio the same as that to be used in the distribution train. Two different methods were used for developing and analysing the concentration profiles: in the first, the absorbance of the upper or lower phase in the distribution units was measured, while in the second, the effluent collected from the apparatus was analysed spectrophotometrically.

The calculations of the theoretical distribution curves and of the partition coefficients were made as reported by Ellfolk and Hynninen[8], according to the theory of Martin and Synge[7].

A Uvichem Model H-1620 spectrophotometer was used to measure single absorbances. Before and after each fractionation experiment, the identities and the purities of the separated compounds were controlled by measuring their UV spectra with a Beckman Model DB-GT instrument and their IR spectra with a Perkin-Elmer 157 instrument, and evaluating their thin-layer chromatographic R_f values on Merck silica gel F_{254}.

Choice of the solvent systems

Phenolic O-glucuronides and phenolic sulphate esters are generally polar compounds, particularly the former because of the high hydrophilicity of the glucuronic acid molecule; moreover, the phenolic O-glucuronides are weak acids (pK_a of glucuronic acid = 5.8), while the corresponding sulphates are strongly acidic. As a result of these properties, the salts of phenolic O-glucuronides and phenolic sulphate esters show, at neutral pH, low partition coefficients between organic and aqueous phases even with good solvents such as *n*-butanol and isoamyl alcohol. A better partition of the phenolic O-glucuronides is possible under moderately acidic conditions (pH 5.0–4.0), whereas lower pH values (3.0–2.0) are required in order to

extract the phenolic sulphates from the aqueous phase. As both phenolic O-glucuronides and phenolic sulphate esters show a higher partition coefficient as free acids, the separation of these conjugates is more easily performed after further transformation from the salt from into the acidic form. In the case of the sulphate esters, the salt–acid conversion is easily accomplished using a strongly cationic exchanger such as Amberlite IR-120 (H^+). For the glucuronides, which are often labile, a rapid acid extraction is preferable.

These considerations, and the physico-chemical properties of the phenolic O-glucuronides and phenolic sulphate esters in the single solvent systems adopted (solubility, reactivity, surface phenomena, etc.), were taken into account when choosing partition systems suitable for their fractionation.

In addition to the required partition properties, the solvent systems selected satisfy other requirements: they are intentionally neutral and virtually inert so as to avoid any possible degradation of the compounds to be fractionated, have a high dissolving capacity as the semi-preparative purposes require, permit UV analysis at wavelengths above 260 nm and, finally, they can be easily modified as necessary for a particular problem.

RESULTS

Taking into account the special difficulties that arise when working with biological material, and in order to evaluate all possible cases, the following types of separations were studied.

(1) Separation of phenolic O-glucuronides (salts) from the corresponding phenolic sulphate esters (salts).

(2) Separation of phenolic O-glucuronides (acids) from the corresponding phenolic sulphate esters (acids).

(3) Fractionation of a mixture of phenolic sulphate esters (salts).

(4) Fractionation of a mixture of phenolic sulphate esters (acids).

(5) Fractionation of a mixture of O-glucuronides (acids).

As reference compounds, the phenolic O-glucuronides and the phenolic sulphates of *p*-nitrophenol and 6-bromo-2-naphthol were used.

Separation of phenolic O-glucuronides (salts) from the corresponding phenolic sulphate esters (salts)

Phenolic O-glucuronides and phenolic sulphate esters are excreted in the urine as salts. Therefore, it is often useful to separate the two classes of conjugates already in this form. For this separation, of the solvent systems studied, one of the most satisfactory was the following:

Sodium phosphate buffer, 0.01 *M*, pH 6.8	4.0 (v/v)	System I
n-Butanol	1.0 (v/v)	
n-Propanol	1.0 (v/v)	
Ethyl acetate	3.0 (v/v)	

The use of a buffered system is necessary in order to avoid dissociation of the phenolic O-glucuronide salts, while *n*-propanol improves the separation between the two phases.

The result of the fractionation, utilising a 70-tube distribution train, is shown in Fig. 1. It can be seen that phenolic sulphate esters are easily eluted from the apparatus with experimental partition coefficients $K_1=4.25$ ($V_m=0.38$ l) and $K_2=1.16$ ($V_m=0.83$ l). V_m= elution volume of the moving phase; on the other hand the corresponding phenolic O-glucuronides show partition coefficients less than 1 ($K_3=0.527$ and $K_4=0.081$), so that their elution from the apparatus will require calculated volumes of moving phase of 1.569 and 8.995 l, respectively.

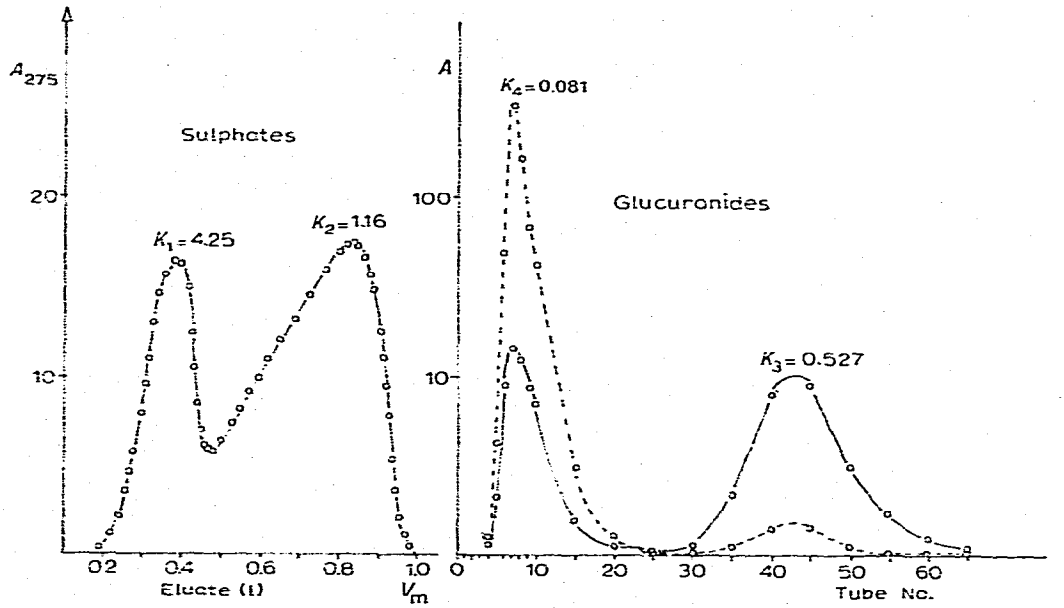


Fig. 1. Separation of a mixture of phenolic O-glucuronides and phenolic sulphate esters in the salt form using solvent system I. K_1=6-bromo-2-naphthyl sulphate; K_2=*p*-nitrophenyl sulphate; K_3=6-bromo-2-naphthyl-β-D-glucuronide; K_4=*p*-nitrophenyl-β-D-glucuronide. A 25-mg amount of each compound was dissolved in 20 ml of the stationary phase and the mixture was loaded into the first four tubes of the distribution apparatus. The number of tubes used in the process was 70. v_m=3.2 ml; v_s=10.3 ml; $z=v_m/v_s=0.31$; V_m=1 l; flow-rate=1 ml/min. Experimental curves obtained by measuring the absorbance, *A*, of the moving phase in the eluate at 275 nm, and of the stationary phase in the distribution train at 275 (— · — · —) and 300 (— — — —) nm.

The partition profile of the phenolic sulphate esters deviates considerably from the theoretical profile. This can probably be attributed to the surfactant properties of these compounds, which result in emulsions that affect the phase ratio of the solvent system.

Separation of phenolic O-glucuronides (acids) from the corresponding phenolic sulphate esters (acids)

The phenolic sulphate esters in their acid form can be easily separated from the corresponding phenolic O-glucuronides by using the following partition system:

Water	2.5 (v/v)	System II
Formamide	2.5 (v/v)	
Diisopropyl ether	3.7 (v/v)	
Benzene	1.0 (v/v)	

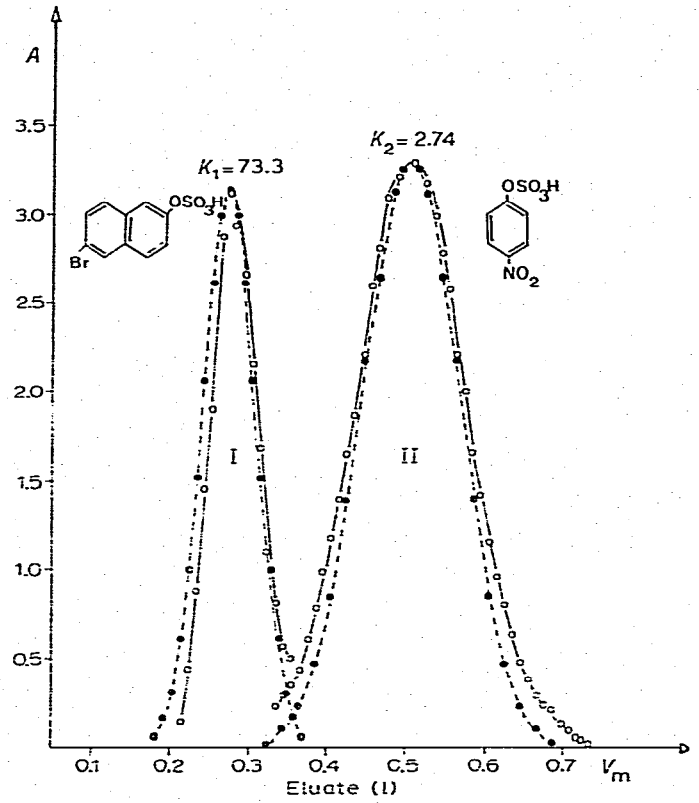


Fig. 2. Separation of phenolic sulphate esters from the corresponding phenolic O-glucuronides in the acid form using solvent system III. K_1 = 6-bromo-2-naphthyl sulphate; K_2 = *p*-nitrophenyl sulphate. A 25-mg amount of each compound was dissolved in 10 ml of the stationary phase and the mixture was loaded into the first four tubes of the distribution apparatus. v_m = 4.0 ml; v_s = 9.5 ml; $z = v_m : v_s = 0.42$; $V_m = 0.75$ l. The number of tubes used in the process was 50. ○, Experimental curve obtained by measuring the absorbance, *A*, of the moving phase at 288 nm (I) and 308 nm (II); ●, theoretical curve.

In this way, the phenolic sulphate esters are rapidly eluted (Fig. 2), while the phenolic O-glucuronides remain in the first tubes of the apparatus. In this case, the deviation from theory of the distribution curve is negligible because in their acid form the phenolic sulphate esters lose their surfactant properties.

Fractionation of a mixture of phenolic sulphate esters (salts)

The surfactant properties of the phenolic sulphate salts complicate their fractionation by liquid–liquid partition. As an example, the separation of the 6-bromo-2-naphthyl sulphate from the *p*-nitrophenyl sulphate is shown in Fig. 3; the solvent system used was as follows:

Sodium phosphate buffer, 0.01 *M*, pH 7.0	4.0 (v/v)	System III
n-Butanol	1.0 (v/v)	
Ethyl acetate	3.0 (v/v)	

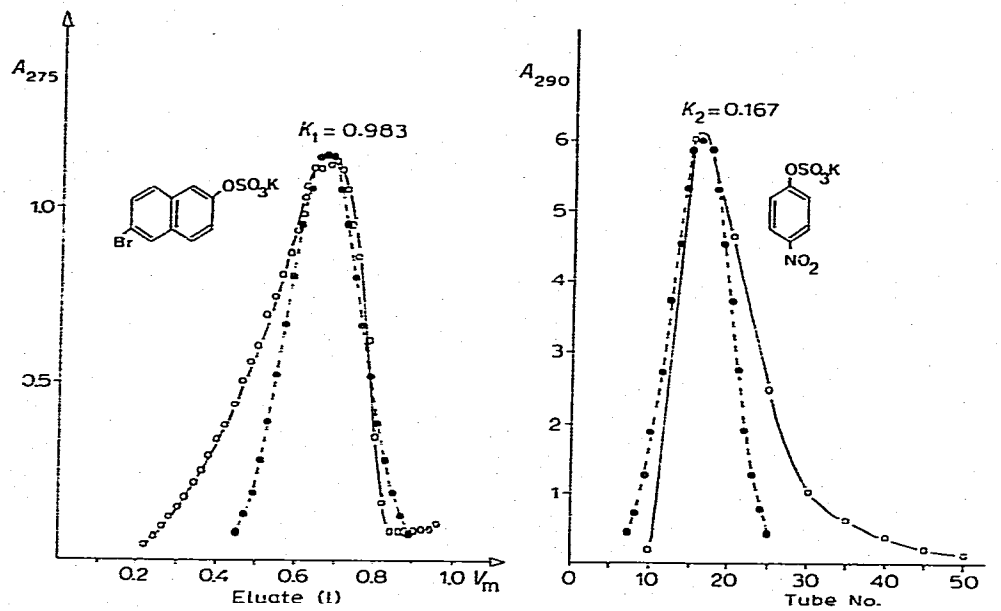


Fig. 3. Separation of phenolic sulphate esters salt mixture using solvent system III. K_1 = 6-bromo-2-naphthyl sulphate; K_2 = *p*-nitrophenyl sulphate. A 25-mg amount of each compound was dissolved in 10 ml of the lower phase and the mixture was loaded into the first two tubes of the distribution apparatus. The number of tubes used in the process was 70. v_m = 3.2 ml; v_s = 10.3 ml; $\alpha = v_m/v_s = 0.36$; V_m = 1 l; flow-rate = 1.5 ml/min. ○, Experimental curve obtained by measuring the absorbance, *A*, of the moving phase at 275 nm and of the stationary phase at 290 nm; ●, theoretical curve.

The partition coefficients of the two sulphates are different (K_1 = 0.983; K_2 = 0.167), resulting in a good resolution, and consequently the fractionation can even be accelerated by adding, for example, small amounts of *n*-propanol.

The expected deviation of the experimental curve results in large tails in front of the peaks, indicating the appearance of the lower phase emulsified with the upper phase.

Fractionation of a mixture of phenolic sulphate esters (acids)

In their acid form, the phenolic sulphate esters are easily extracted from the aqueous phase, and a moving phase with low polarity can be used for the partition. For example, solvent system IV gave a good fractionation of the sulphates studied, in agreement with the theoretical result (Fig. 4).

Water	3.0 (v/v)	System IV
Formamide	2.0 (v/v)	
Diisopropyl ether	3.0 (v/v)	
Benzene	1.4 (v/v)	

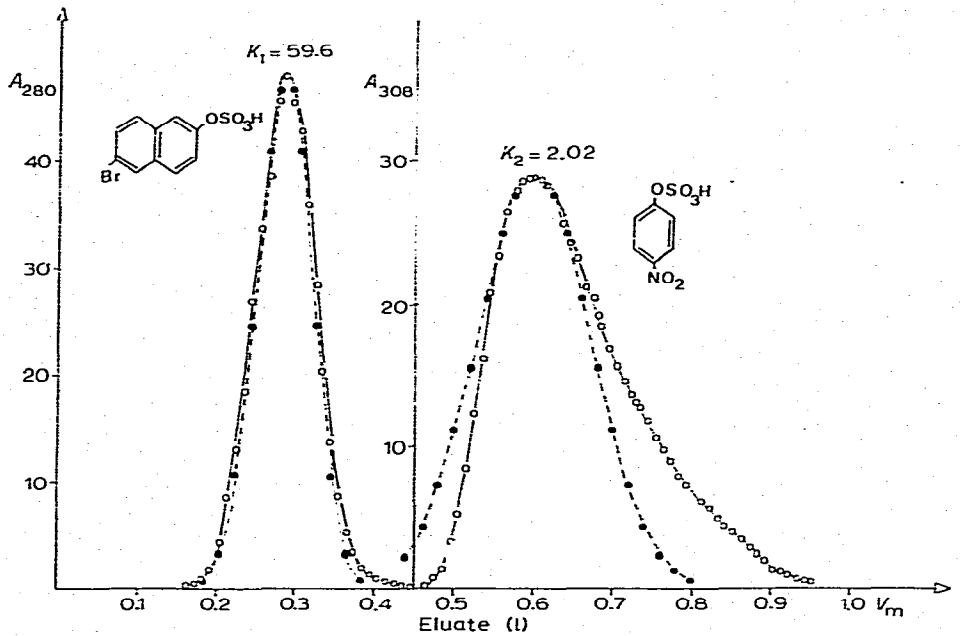


Fig. 4. Separation of phenolic sulphate ester mixture (acid form) using solvent system IV K_1 = 6-bromo-2-naphthyl sulphate; K_2 = *p*-nitrophenyl sulphate. A 25-mg amount of each compound was dissolved in 10 ml of the stationary phase and the mixture was loaded into the first two tubes of the distribution apparatus. The number of tubes used in the process was 70. v_m = 4.0 ml; v_s = 9.5 ml; $\alpha = v_m/v_s = 0.42$; V_m = 1 l; flow-rate = 1.5 ml/min. ○, Experimental curve obtained by measuring the absorbance, *A*, of the moving phase at 280 and 308 nm; ●, theoretical curve.

This solvent system can be further improved by lowering the partition coefficients of the components being fractionated by decreasing the water:formamide ratio or the diisopropyl ether:benzene ratio.

Fractionation of a mixture of phenolic O-glucuronides (acids)

Of the solvent systems considered, the following was the most satisfactory:

Water	3.7 (v/v)	System V
N,N-dimethylformamide	1.2 (v/v)	
n-Butanol	0.7 (v/v)	
Diisopropyl ether	1.0 (v/v)	
Ethyl acetate	2.3 (v/v)	

This system shows a high resolution capacity, which can be increased by decreasing the water:N,N-dimethylformamide ratio or the ethyl acetate:diisopropyl ether ratio. The deviation of the experimental curve from the theoretical curve (Fig. 5) is attributed only to the low value of the phase ratio, which changes little during the partition.

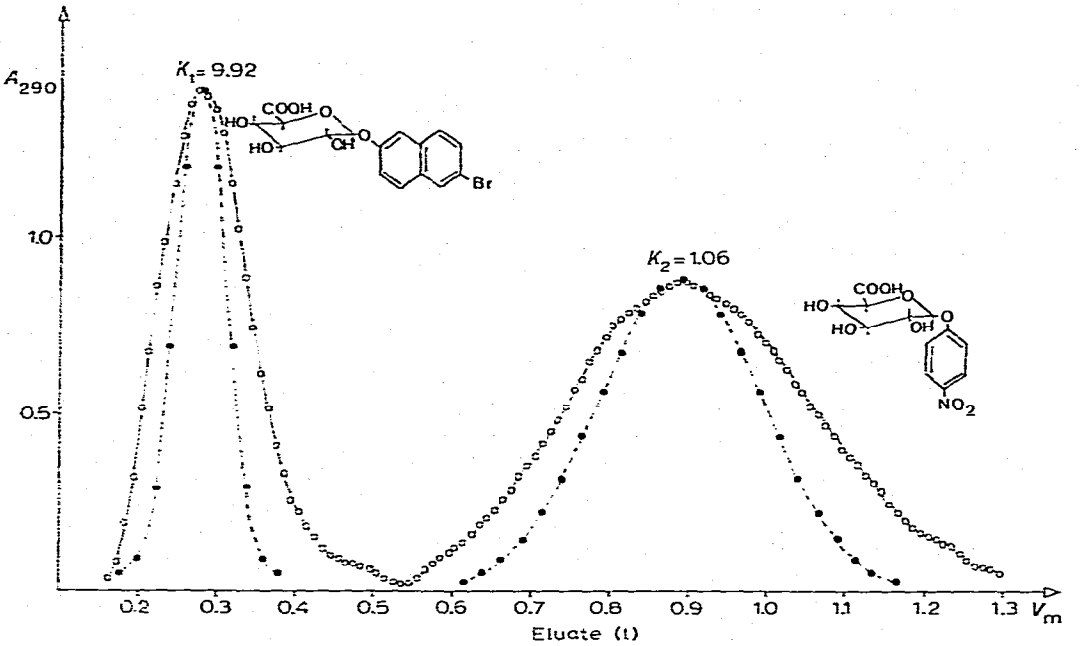


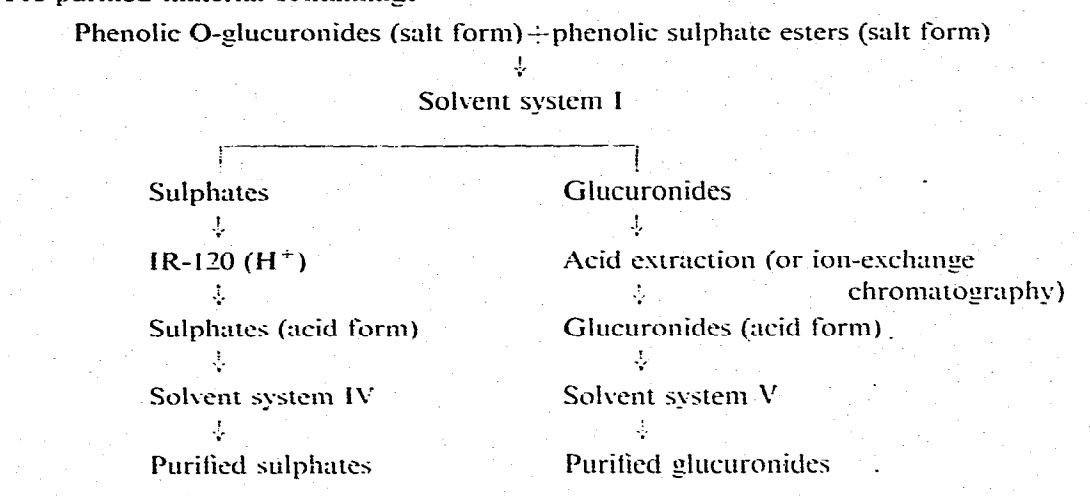
Fig. 5. Separation of a phenolic O-glucuronide mixture (acid forms) using solvent system V. K_1 = 6-bromo-2-naphthyl-β-D-glucuronide; K_2 = *p*-nitrophenyl-β-D-glucuronide. A 25-mg amount of each compound was dissolved in 10 ml of the stationary phase and the mixture was loaded into the first two tubes of the apparatus. The number of tubes used in the process was 70. v_m = 3.0 ml; v_s = 10.5 ml; $z = v_m/v_s$ = 0.286; V_m = 1.3 l; flow-rate = 1 ml/min. . Experimental curve obtained by measuring the absorbance, *A*, of the moving phase at 290 nm; ●, theoretical curve.

DISCUSSION

The results obtained demonstrated the possibility of separating by multiple liquid–liquid partition phenolic O-glucuronides from the corresponding phenolic sulphate esters and the different compounds within the two classes of conjugates.

Obviously, the isolation of these compounds from biological fluids (urine and bile) requires pre-purification of the crude material[3] before the countercurrent fractionation; in fact, of the various impurities present in urine or bile, salts interfere with the partition systems, affecting the ratio between the two phases, and the other contaminants may affect the analytical measurements. The choice of the analytical method to be used to follow the partition profiles is particularly difficult when the compounds to be isolated have low molar extinction coefficients at wavelengths above 280 nm, because in the far UV region interferences by impurities and by the solvent systems used are very high.

These difficulties can be prevented by the use of labelled compounds. Both from our earlier experience and the results of the present work, we suggest the following scheme for isolating phenolic O-glucuronides and phenolic sulphate esters from biological fluids.



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