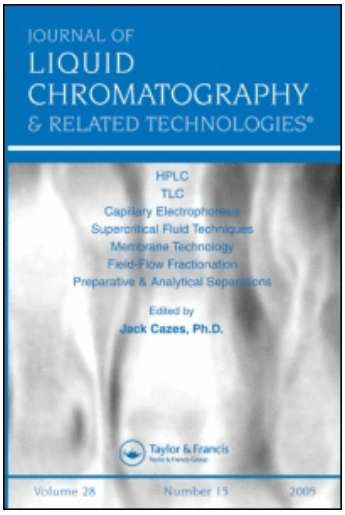

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Evaluation of Nonaqueous Solvent Systems for Countercurrent Chromatography Using an HPLC Assay to Determine Partition Coefficients of a Mixture of Compounds

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EVALUATION OF NONAQUEOUS SOLVENT SYSTEMS FOR COUNTERCURRENT CHROMATOGRAPHY USING AN HPLC ASSAY TO DETERMINE PARTITION COEFFICIENTS OF A MIXTURE OF COMPOUNDS

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ABSTRACT

A reversed phase HPLC assay was used to measure partition coefficients for salicylic acid, salicylamide and salicyluric acid in two-phase systems employing neat or aqueous ethylene glycol, formamide, or methanol and the organic solvents: ethylene dichloride, ethyl acetate, diethyl ether and methyl isobutyl ketone. These solvent systems permit preparative separation by countercurrent chromatography of compounds with limited aqueous solubility. Salicylic acid, salicylamide and salicyluric acid are readily separated in the system employing ethylene glycol as the stationary phase and diethyl ether as the mobile phase.

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INTRODUCTION (1,2)

Selection of solvent systems for the separation of compounds by countercurrent chromatography is expedited by a knowledge of the partition coefficients of the solutes in the various solvent systems. Partition paper chromatography (3) and TLC (4,5) have been suggested as means for surveying possible solvents for separation of mixtures by liquid-liquid partition; but errors may arise in these methods as a result of unpredictable adsorption to the supporting matrix. The approach described here involves partition of a mixture of solutes between small volumes of immiscible liquid phases and subsequent measurement of the concentration of solutes in each phase using reversed phase liquid chromatography.

Because of their strong solubilizing properties, the polar solvents ethylene glycol (EG) and formamide (F) as well as 80% EG in water and 80% methanol (M) in water are examined for possible use as stationary phases for countercurrent chromatographic separation of compounds having relatively low aqueous solubility. A mixture of salicylic acid (SA), salicylamide (SAM) and salicyluric acid (SU) is employed as a test mixture. The solubilities of SA and SAM and the partition coefficients of these and SU are determined in two-phase systems consisting of the above solvents and methylene dichloride (MDC), ethylene dichloride (EDC), ethyl acetate (ETAC), diethyl ether (ET) and methyl isobutyl ketone (MIK).

EXPERIMENTAL

Reagents

Methanol was HPLC grade. All other chemicals were reagent grade.

Apparatus

Countercurrent chromatography was done with a horizontal flow-through coil planet centrifuge (6,7) using the planet gear drive at 400 rpm, β 0.25 and a column consisting of 5 m of 2.6 mm ID PTFE tubing wound (98 turns) on a 12.5 mm rod. Column volume was 25 ml. Solvent was delivered with a Beckman Accuflow pump. Column effluent was monitored at 254 nm using an LKB Uvicord S monitor (LKB Instruments Inc., Rockville, MD) with a 1.8 mm cell and an LKB model 6520 recorder.

HPLC was done using a modular system consisting of a Glenco HPLPS-1 pump, Glenco SV-3 injection valve with 50 µl loop, Glenco 5480 monitor (254 nm) and an Alltech C-18 column (10 µ, 4.6 x 250 mm). Samples (25 µl) were injected using a Valco 50-µl syringe.

Methods

HPLC. Chromatographic separation of SA, SAM and SU was examined using mobile phases composed of 0.05 M Na formate buffer:MeOH; 70:30 (v/v) with buffers of pH 3.00, 3.25, 3.50 and 4.00. Routine chromatography employed the pH 3.25 buffer. Flow-rate was 1 ml/min and monitoring was at 254 nm. Calibration plots were prepared by assay of 20-, 50-, 100-, and 200-fold dilutions in mobile phase of stock solutions containing 4, 8 and 2 mg/ml of SA, SAM and SU respectively.

Partition coefficients. Two ml of a stock solution in ethylene glycol (or formamide or aqueous methanol) containing 4, 8 and 2 mg/ml of SA, SAM and SU respectively, was gently mixed with 2 to 5 ml of a second, immiscible solvent for 30 min at room temperature in a 15-ml graduated centrifuge tube closed with a teflon-lined cap. After centrifugation, 10- to 50-fold dilutions of each layer were prepared in HPLC mobile phase using volumetric flasks and 25- to 250-µl Kirk transfer pipets. Aliquots of solvents (EDC, etc.) not miscible with the mobile phase were evaporated in an air stream and the residue diluted with

mobile phase. Aliquots (25 µl) of diluted solutions were assayed by HPLC using the pH 3.25 mobile phase. Retention times for SU, SAM and SA were 10, 11.2 and 15.2 min respectively. Partition coefficients were calculated as

$$K_{u/l} = \frac{h_u \, d_l}{h_l \, d_u}$$

where h_u, h_l, d_u amd d_l represent the peak heights and dilutions of the upper and lower layers respectively. To minimize error, dilutions were chosen to provide approximately equal values of h_u and h_l.

Solubilities. Saturated solutions of SA and SAM in several solvents were prepared by allowing an excess of compound in 1 ml of solvent in a capped vial to remain for several days at room temperature with intermittent mixing. Aliquots of the supernatant liquid were measured with Kirk microliter pipets, diluted with HPLC mobile phase (pH 3.25) and assayed by HPLC.

Countercurrent Chromatography. Retention of ethylene glycol when using ethyl acetate and diethyl ether as mobile phases was measured for various flow rates at β 0.25 and various rates of revolution by observation, under stroboscopic illumination, of the number of coils occupied by 5.0 ml of ethylene glycol (ethyl acetate or ether-saturated) dyed with acid fuchsin (8). The fraction of column volume, F_S, occupied by stationary phase is then given by

$$F_S = \frac{v_S}{n \, v_C}$$

where v_S is the volume of dyed stationary phase introduced, v_C is the volume of a single coil of the column and n is the number of coils containing dyed stationary phase under the particular conditions of flow-rate, rate of rotation and β.

Solvent systems for separation by countercurrent chromatography were prepared by mutually saturating ethylene glycol and ethyl acetate or diethyl ether in a separatory funnel. After filling the column by pumping in the upper phase at a rate

of 1.35 ml/min, the apparatus was rotated at 400 rpm and exactly 15 ml of solvent-saturated ethylene glycol introduced by means of a loop injector. The stationary phase volume, V_S, was calculated by subtracting the unretained portion of the stationary phase as measured by collecting the column effluent in a 25-ml graduated cylinder. The difference between the stationary phase volume and the total column volume (25 ml) is the mobile phase volume (V_m). An 0.5-ml aliquot of an ethylene glycol solution containing 1.1, 3.0 and 2.5 mg/0.5 ml of SU, SAM and SA respectively, was injected using a sample loop of 18 gauge (1.02 mm ID) PTFE tubing. Effluent was monitored at 280 nm with an LKB monitor using a 1.8 mm cell.

The number of theoretical plates, N, for each solute was calculated using the conventional formula

$$N = 16\left(\frac{t_R}{w}\right)^2$$

where t_R is the retention time and w the peak-width at the base.

The partition coefficient, $K_{s/m}$, was calculated from the capacity factor, k', by multiplying by the phase volume ratio

$$K_{s/m} = k'\left(\frac{V_m}{V_s}\right) = \frac{t_R - t_m}{t_m}\left(\frac{V_m}{V_s}\right)$$

where V_m and V_s are the volumes of mobile and stationary phases respectively and t_R and t_m are the retention times and holdup times respectively.

RESULTS AND DISCUSSION

HPLC

Evaluation of k' as a function of mobile phase pH, Fig. 1, indicated that a mobile phase composition of 0.05 M Na formate buffer, pH 3.25:MeOH; 70:30 (v/v) provided satisfactory resolution of SU, SAM and SA as shown in Fig. 2. Peak heights were linearly related to injected concentrations over the concentration ranges

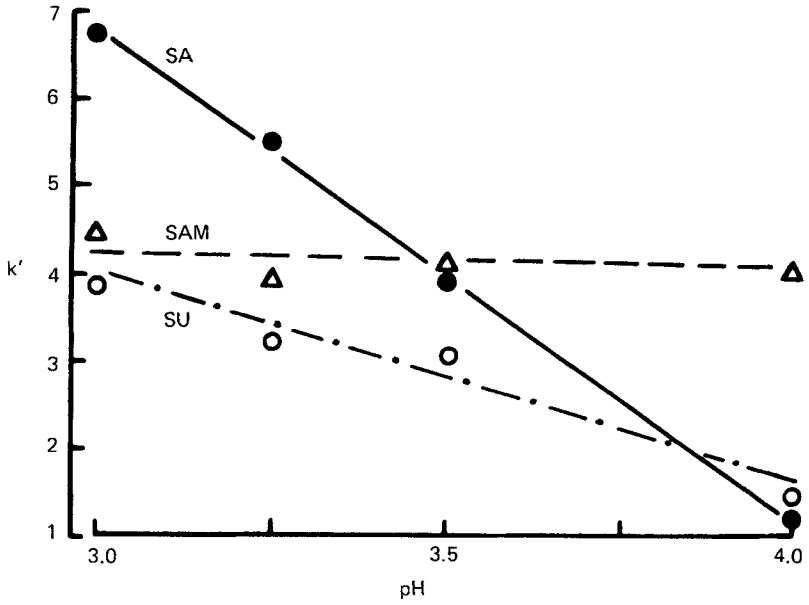

FIGURE 1. HPLC Capacity factor, k´, as a function of pH of mobile phase consisting of 30% methanol in 0.05 M sodium formate buffer.

employed: 10-100 µg/ml for SU, 40-400 µg/ml for SAM and 20-200 µg/ml for SA with correlation coefficients (3 injections at each of 4 concentrations) of 0.9995, 0.994 and 0.998, respectively.

Solubilities

Solubilities of SA and SAM in several solvents and solvent mixtures, most of which were nonaqueous, are summarized in Table 1. The solubilities are appreciable in most of the solvents. While the solubility of SU was not measured, preparation of several saturated solutions showed that it is more soluble than either SA or SAM in most of these solvents. It should also be noted that mutual saturation of two immiscible solvents, such as ETAC/EG, results in a significant increase in the solubilities of SA and SAM compared with their solubilities in the neat solvents.

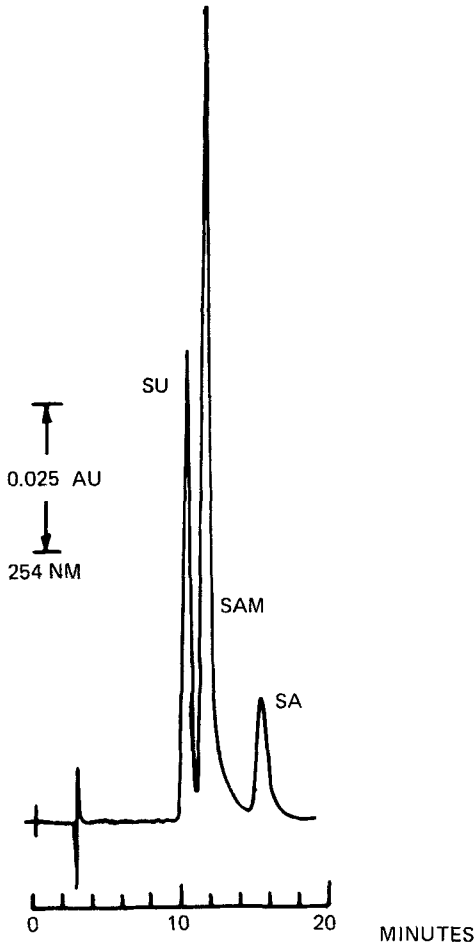

FIGURE 2. HPLC separation of salicyluric acid (SU), salicylamide (SAM) and salicylic acid (SA) on a C-18 column with mobile phase composed of 0.05 M sodium formate buffer: methanol; 70:30 (v/v), pH 3.25 at a flow rate 1 ml/min. Sample is 25-µl of an ethylene glycol stock solution containing 2, 8 and 4 mg of SU, SAM and SU, respectively, which was diluted 1/20 with mobile phase.

TABLE 1

Solubilities of Salicylic Acid and Salicylamide at Room Temperature

Solvent	Solubility, mg/ml	
	Salicylic Acid	Salicylamide
Ethyl Acetate	164	111
80% Ethyl Acetate in Ether	192	100
50% Ethyl Acetate in Ether	208	77
Diethyl Ether	283	32
Methyl iso-Butyl Ketone	186	103
80% Methanol	206	79
Chloroform	38	8
Ethylene Dichloride	21	22
Ethylene Glycol	120	105
80% Ethylene Glycol in Water	60	53
Formamide	230	118
1% Acetic Acid in Formamide	188	140
1% Acetic Acid in 60% Formamide in Water	296	40
Ethyl Acetate/Ethylene Glycol		
upper phase	270	166
lower phase	283	150

Partition Coefficients

The partition coefficients of SA, SAM and SU in several solvent systems are summarized in Table 2. The relative solvent volumes used in the determination are indicated. For systems containing three or more solvents, the partition coefficient may be expected to vary with the relative solvent volumes employed, since the solvents were not mutually saturated prior to equilibration. This potential variation must be taken into account when preparing large volumes of solvent for use in countercurrent chromatography.

Countercurrent Chromatography

The systems ETAC/EG and ET/EG were examined for use in countercurrent chromatography with EG as the stationary phase.

TABLE 2

Partition Coefficients Determined by HPLC

Solvents (2) A	B	Vol. B[2] / Vol. A	Partition Coefficient C_A/C_B SA	SAM	SU
83% EG[1]	MDC	2.1	5.2	5.4	>20
EG	MDC	1.5	4.7	4.2	>20
EG	EDC	2.5	12	12	>20
80% EG[1]	ETAC	1	0.19	0.50	1.19
EG	ETAC	2.5	0.59	0.92	2.48
EG	ET	2.5	0.65	4.36	13.5
EG	50% ETAC in ET	1	0.47	1.69	4.17
EG	80% ETAC in ET	1	0.61	1.30	2.85
EG	MIK	2.5	0.47	0.89	2.53
F	ET	2.5	>20	4.35	>20
1% HOAc in F	ET	1	>20	3.57	>20
1% HOAc in 60% F[1]	ET	1.3	3.70	1.31	16.7
1% HOAc in 60% F[1]	ETAC	1.3	4.00	0.39	5.0
F	EDC	2.5	>20	5.00	>20
80% M[1]	EDC	2.5	0.75	1.45	2.7

[1] in water
[2] relative volume ratio before equilibration

As summarized in Table 3, the amount of EG retained varies from 28 to 44% and 38 to 49% of column volume, with ETAC and ET respectively as mobile phase over a wide range of flow rate and rate of revolution of the column holder.

As expected from the partition coefficients (Table 2), countercurrent chromatography, using ETAC/EG on a column consisting of a 5 m length of 2.6 mm i.d. PTFE tubing wound as 98 turns on a 12.5 mm core, did not resolve SA and SAM, although both were reasonably separated from SU.

The three compounds were fully resolved by countercurrent chromatography in the ET/EG system as shown in Fig. 3. The the-

TABLE 3

Retention of Ethylene Glycol as Stationary Phase Using Either Ethyl Acetate or Diethyl Ether as Mobile Phase with Planet Gear Drive and β 0.25

Mobile Phase Flow Rate, ml/min	Revolutional Rate, RPM	Stationary Phase Retention, F_S	
		ETAC	ET
0.62	300	0.43	0.49
	400	0.44	0.48
	600	0.43	0.48
1.30	300	0.38	0.43
	400	0.40	0.44
	600	0.40	0.44
1.95	300	0.33	0.40
	400	0.36	0.42
	600	0.36	0.43
2.74	300	0.28	0.38
	400	0.32	0.40
	600	0.34	0.41

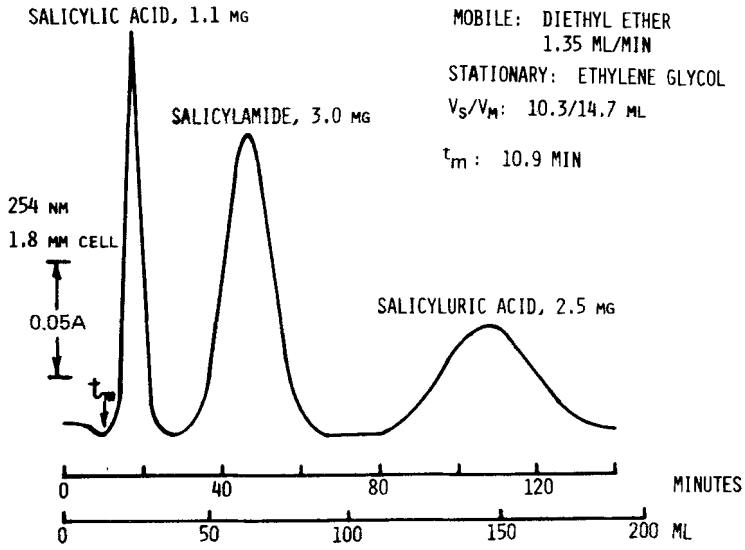

FIGURE 3. Countercurrent chromatographic separation of SA, SAM and SU.

oretical plate numbers, N, were 32, 48 and 79 for SA, SAM and SU respectively. The partition coefficients, 0.74, 4.34 and 12.7, calculated from the capacity factors, k', 0.52, 3.04 and 8.91 for SA, SAM and SU respectively, are in good agreement with the values measured by HPLC (Table 2).

The high boiling point of EG mitigates against its use as a mobile phase in countercurrent chromatography; however, its solubilizing properties recommend its use as a stationary phase. In preparative applications solutes recovered by evaporation of the mobile phase will be contaminated with ethylene glycol. This may often be removed simply by washing with water. In instances where water is undesirable, azeotropic distillation with toluene or heptane may be employed to remove ethylene glycol at a moderate temperature (9).

Conclusion

HPLC is a useful means for measuring partition coefficients for evaluation of solvent systems for countercurrent chromatography, particularly in those instances where UV-absorbing solvents are to be considered, and where only a mixture, such as a natural product extract, is available for evaluation. Where pure solutes are available, and the search is confined to UV-transparent solvents, development of an HPLC assay may be unduly time consuming, and use of a previously described simple partitioning system will usually be faster (10).

Because of their good solubilizing properties and immiscibility with a number of moderately polar solvents, ethylene glycol and formamide may be advantageously employed as stationary phases in countercurrent chromatography.

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2. Abbreviations used: TLC, thin-layer chromatography; EG, ethylene glycol; F, formamide; M, methanol; SA, salicylic acid; SAM, salicylamide; SU, salicyluric acid; MDC, methylene dichloride; EDC, ethylene dichloride; ETAC, ethyl acetate; ET, diethyl ether; MIK, methyl isobutyl ketone; PTFE, polytetrafluoroethylene; HPLC, high pressure liquid chromatography; $K_{u/l}$, $K_{s/m}$, partition coefficient expressed the ratio of concentrations in upper and lower layers or stationary and mobile phases respectively; k´, capacity factor.

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