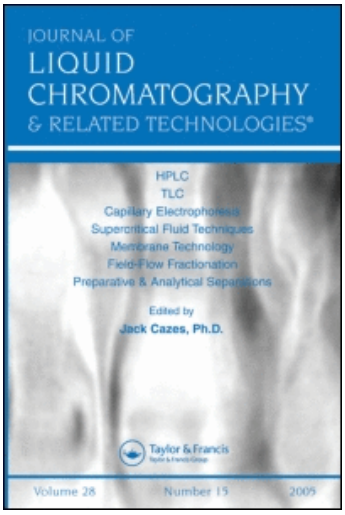

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Conway, Walter D. , Yeh, Hsing C. and Tedesche, Nicholas J.(1992) 'Evaluation of Heptane/1-Butanol/Water Systems for Separation of Drug Metabolites by Countercurrent Chromatography', Journal of Liquid Chromatography & Related Technologies, 15: 15, 2751 — 2768
To link to this Article: DOI: 10.1080/10826079208016346
URL: http://dx.doi.org/10.1080/10826079208016346

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(15&16), 2751-2768 (1992)

EVALUATION OF HEPTANE/1-BUTANOL/WATER SYSTEMS FOR SEPARATION OF DRUG METABOLITES BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

A small-volume CCC coil (1.07 mm i.d. x 13.5 m, V_c 14.7 ml) has been used to evaluate heptane/1-butanol/aqueous buffer systems for potential use in separating drug metabolites. Repeated sample injections can be made and a partition coefficient as high as 40 can be determined in about 4 hrs. Conjugates of p-nitrophenol with glucuronic acid, sulfuric acid and glucose were separated, and the partition coefficients of several N-alkyl anilines, benzylamines and phenylethylamines were measured to emulate chromatographic behavior expected for compounds generated in xenobiotic metabolism. Amines chromatographed well in reverse phase systems above the amine pK_a value. But peaks were distorted when the mobile phase pH was slightly below the amine pK_a for benzyl and phenylethyl amines and about two units below for anilines. The effect of alcoholic hydroxyl substitution and dealkylation is discussed for anilines and derivatives of the drug chloroquine.

INTRODUCTION

The attributes of total sample recovery, choice of normal or reverse phase mode, operation over the entire pH range, and acceptance of both polar and nonpolar solutes recommend countercurrent chromatography (CCC) for the separation and isolation of drug metabolites from biological fluids. The wide range of solute polarity between the parent drug and metabolites precludes resolution of all analytes in a single isocratic system, but stepwise or gradient elution using heptane/n-butanol/water systems would provide a wide range of organic phase polarity.

MATERIALS AND METHODS

Solvents and other chemicals were HPLC grade or reagent grade. Conjugates of p-nitrophenol were obtained from Sigma Chemical Co.

Ternary Diagram

The binodal curve for the heptane/1-butanol/water system was determined by turbidity titration using class A burets at a room temperature of 20°C. Solutions containing 10, 20, 30, 40, 50, 60, 70, 80 and 90% 1-butanol, by volume, in heptane were prepared using volumetric pipets and flasks. To determine the tie-lines, aliquots (5-ml) of these solutions, as well as neat heptane and neat 1-butanol were equilibrated with 5 ml of water in calibrated, screw cap, 15-ml centrifuge tubes and equilibrated by mixing for one hour on a wrist-action shaker followed by centrifuging for 30 minutes at 1000 rpm. Phase volumes were recorded and the phases separated. Composition of the lower phase was determined by refractometry (Bausch & Lomb Abbe-3L refractometer) at 20°C using standards prepared by dissolving 1-butanol in water. Tie lines were drawn through this point and the ternary composition point and extended to intercept the solubility curve on the ternary diagram, Fig. 1. The composition of this intercept

was taken as the composition of the upper (organic) phase. The volumetric solubilities of 1-butanol in water (9.5%) and of water in 1-butanol (17.5%) were calculated from the published solubilities by weight (7.81 and 20.7 g/100 g respectively and the density of 1-butanol (0.810 g/ml).[2] Values in Table 1 are rounded to the nearest 0.5%.

Apparatus

The CCC system, Fig. 2., included a P.C. Inc. multilayer coil planet centrifuge equipped with a TRIPPLE™ Coil containing a 1.07 mm x 13.7 m (14.7 ml) coil at ß of 0.85, operated at 1200 rpm, and a Milton-Roy model 196-31 pump, a Valco C6-P injection valve, an Isco V-4 absorbance monitor with a 2-mm pathlength prep. cell, and a Houston Instrument Omniscribe chart recorder.

The volume of stationary phase displaced was collected in a carryover vessel constructed from a modified 10-ml volumetric pipet and could be estimated to 0.01 ml. The vessel was oriented as shown to collect a lighter stationary phase and can be inverted to collect the heavier phase. Extra-column feed lines of 0.5 mm i.d. tubing resulted in a volume, $V_{f,D}$, between the injection port and the detector of 0.58 ml and an additional volume, $V_{f,DC}$, after the detector, of 0.17 ml. These volumes could be reduced significantly by employing 0.3 mm i.d. tubing but at the expense of significantly increased backpressure both during chromatography and when manually loading stationary phase.

CCC Procedure

Stationary phase was manually loaded into the motionless coil through the loading port S, Fig. 2, prior to attachment of the carryover vessel. The carryover vessel, filled with mobile phase, was then attached. Rotation was then adjusted to approx. 1200 rpm in the forward direction and the heavier, aqueous phase pumped onto the column in the central to peripheral, head to tail, (H) → T, direction. Additional details of operation are indicated in the Figures.

Calculations

When added to half the injection volume, $V_{i/2}$, the pre-detector extra-column volume, $V_{f,D}$, leads to a peak displacement volume, $V_{d,D}$, given by

$$V_{d,D} = V_i/2 + V_{f,D} \quad (1)$$

which amounts to 0.62 or 0.67 ml for injection volumes of 0.1 and 0.2 ml respectively. These amount to about 4% of the column volume, V_c, of 14.7 ml, but are easily corrected for. Peak displacement time, $t_{d,D}$, will be given by dividing the displacement volume by the flow rate, f.

$$t_{d,D} = V_{d,D}/f \quad (2)$$

The chromatographic partition coefficient, K, is defined as

$$K = C_s/C_m = (t_R - t_0)/(t_1 - t_0) \quad (3)$$

where C_s and C_m are the concentrations of solute in the stationary and mobile phases respectively, t_R is the solute retention time and t_0 and t_1 are the retention times for hypothetical solutes with retention times of 0 and 1 respectively. In the reverse phase systems employed here, $K = K_N$, where

$$K_N = C_N/C_A \quad (4)$$

in which C_N and C_A are the solute concentrations in the nonaqueous and aqueous phases respectively.

When a suitable marker is available, the t_0 time is indicated by emergence of the marker peak, $t_{0,0}$. With mobile aqueous phases, we have used both a polymeric dye, Poly R-478 (Sigma) and 2,4-dinitrophenyltaurine, K salt, (DNPTK) (from 2,4-dinitrochlorobenzene and taurine by the method of James and

Synge.[6]) as markers. Poly R-478 is unsatisfactory with heptane, because its peak is broad, and it emerges later than DNPTK. In neat 1-BuOH or heptane/1-butanol mixtures containing predominantly butanol, Poly R-478 emerges first and corresponds closely with $t_{0,co}$ estimated from carryover of stationary phase. However, with alkaline mobile phase, Poly R-478 is delayed slightly; for instance, at pH 8.64, K for Poly R-478 is about 0.05 with 1-butanol as stationary phase. Conversely, below pH 3, DNPTK is delayed slightly.

To judge the suitability of markers, or to estimate K in the absence of a marker, $t_{0,co}$ can be calculated as

$$t_{0,co} = (V_{co} - V_{f,co} + V_{d,D})/f \tag{5}$$

where V_{co} is the volume of stationary phase "carried over" and collected in the carryover vessel, Fig. 2, and $V_{f,co}$ is the extra-column feed-line volume, which was initially filled with stationary phase, from the point of injection to the entrance of the carryover vessel.

The reference point t_1, where a solute having K=1 will emerge is given by

$$t_1 = t_{d,D} + V_c/f \tag{6}$$

where V_c is the column volume.

Additional reference points where solutes with higher partition coefficients will emerge are calculated as

$$t_K = t_0 + K(t_1 - t_0) \tag{7}$$

The fraction of column volume containing stationary phase, S_F, can be estimated as

$$S_F = (t_1 - t_0)/t_c \tag{8}$$

where $t_c = V_c/f$ is the time required for a column volume of mobile phase to flow through the column.

The quantity t_0 in equations 3, 7 and 8 can be estimated from a marker, $t_{0,0}$, or from carryover of stationary phase, $t_{0,co}$, as discussed above.

RESULTS AND DISCUSSION

Ternary Diagram

The ternary phase diagram for the heptane/1-butanol/water system is presented in Fig. 1. A summary of phase compositions is given in Table 1. The phase compositions are very similar to those estimated for the corresponding hexane/1-butanol/water system.[3] The pseudo-two-component notation employed in this table and other figures has been discussed in detail with respect to other heptane/alcohol/water systems and will be presented only briefly here.[4,5]

All solvent systems with composition points lying on the same tie line yield the same conjugate phases, the compositions of which are found at the intersections of the tie line and the binodal curve. These compositions are summarized in Table 1 for selected tie lines. The relative volume of each phase obtained will depend on the location of the point representing the overall ternary composition on the tie line. Combining the composition of the two miscible components, 1-butanol and heptane, permits the use of a simpler notation, that has been called pseudo-two-component notation, which facilitates tabulation of the phase compositions and clarifies discussion of the effect of solvent composition on chromatographic retention.

For instance, the ternary composition heptane/1-butanol/water-2:3:5 (v/v/v) can equally well be expressed in percentage terms as 20:30:50 (v/v/v), or as 30% 1-butanol-in-heptane/water-1:1 (v/v) where the solution of butanol in heptane is regarded as a single component. Ternary compositions corresponding to solutions of 1-butanol in water, mixed with equal volumes of water, lie on the line in Fig. 1 parallel to the right side of the triangle and passing through the points

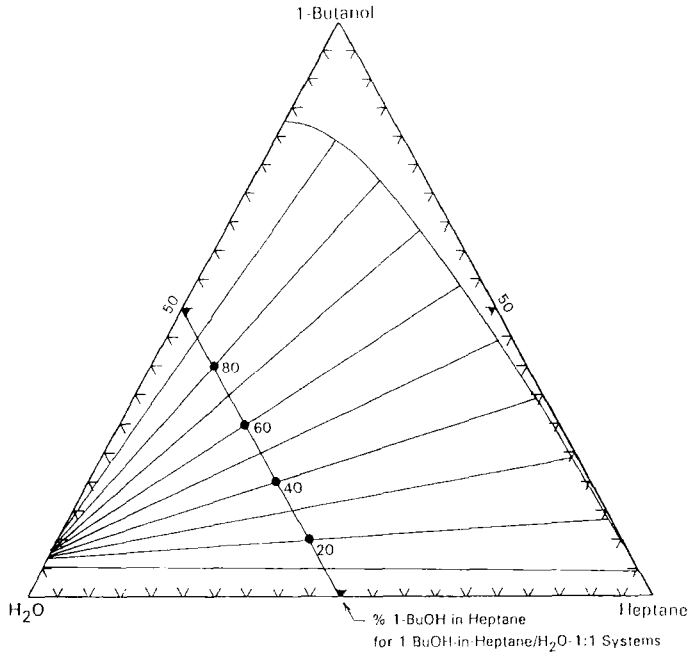

FIGURE 1. Phase diagram for heptane/1-butanol/water (v/v/v), 20°C.

heptane/water-50:50 (i.e. 0% 1-butanol-in-heptane/water) and 1-butanol/water-50:50 (i.e. 100% 1-butanol/water) on the base and left side of the triangle respectively. Compositions on this line will cover the full range of polarity available from this solvent system.

When small amounts of solvent are needed, as for screening partition coefficients by non-chromatographic methods, it is convenient to make them by preparing a series of 1-butanol in water solutions and equilibrating them with equal volumes of water.

Larger volumes, for preparative chromatographic use are better prepared using Table 1. Compositions for both phases are given in percentage terms. After deciding on the relative volumes desired for each phase, the quantities in the table can be multiplied appropriately and added together to give the volumes to

Table 1. Phase Compositions of 1-BuOH-in-Heptane/H_2O-1:1 (v/v) Systems at Approx. 20°C

% 1-BuOH in Heptane	f_a^f From Fig.1	f_a^f Meas. Directly	Conjugate Phase Composition by Volume: Aqueous Phase Heptane	Aqueous Phase 1-BuOH	Aqueous Phase H_2O	Organic Phase Heptane	Organic Phase 1-BuOH	Organic Phase H_2O
0	0.50	0.50	0	0	100	100	0	0
10	0.53	0.54	0	5.5	94.5	95.0	4.5	0.5
20	0.52	0.54	0	6.5	93.5	86.0	13.5	0.5
30	0.53	0.54	0	7.0	93.0	75.0	24.0	1.0
40	0.53	0.55	0	7.0	93.0	64.0	34.5	1.5
50	0.53	0.54	0	7.5	92.5	53.0	44.5	2.5
60	0.52	0.53	0	7.5	92.5	42.0	54.0	4.0
70	0.49	0.52	0	8.0	92.0	30.5	64.0	5.5
80	0.50	0.52	0	8.0	92.0	20.0	72.0	8.0
90	0.49	0.49	0	8.5	91.5	9.5	79.5	11.0
100	0.43	0.43	0	9.5	90.5	0	82.5	17.5

f_a^f = final aqueous fraction after equilibration of initial volumes of solutions of BuOH-in-heptane and water in a 1:1 ratio.

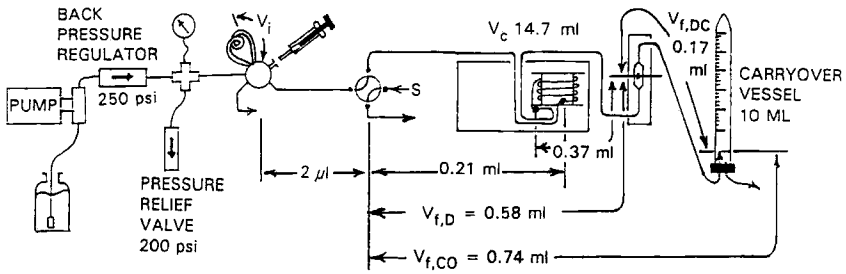

FIGURE 2. Apparatus diagram showing extra-column feed line volumes using 0.5 mm i.d. feed lines. A flow switching valve, Upchurch V-101D, allows manual loading of stationary phase through port S with a 20-ml syringe.

be mixed. For instance, to prepare 200 ml of aqueous phase and 500 ml of organic phase for the 30% 1-butanol-in-heptane/water-1:1 system one would require 5(75) ml heptane, 2(7)+5(24) ml 1-butanol and 2(93)+5(1) ml water, or 375 ml heptane, 134 ml 1-butanol and 191 ml water. On mixing in a separatory funnel, these relative volumes will form 200 ml of aqueous phase and 500 ml of organic phase with compositions corresponding to the 30% 1-butanol-in-heptane/water-1:1 system. Only the desired quantities need be prepared. If, in the course of chromatography, more mobile phase needed, the single phase can be prepared and shaken with some of the excess stationary phase prepared initially to ensure equilibration of the two phases.

Using pseudo-two-compartment notation, the effect of solvent system composition on the partition coefficient, K, is readily illustrated by a plot of K vs % 1-butanol in water for a series of 1:1 systems, which will correspond to tie lines of increasingly polar systems.

Relative Polarity of Heptane/1-BuOH/H_2O Systems

Partition coefficients for several N-alkylbenzamides were determined by CCC and are summarized in Fig. 3 to illustrate the effect of increasing the

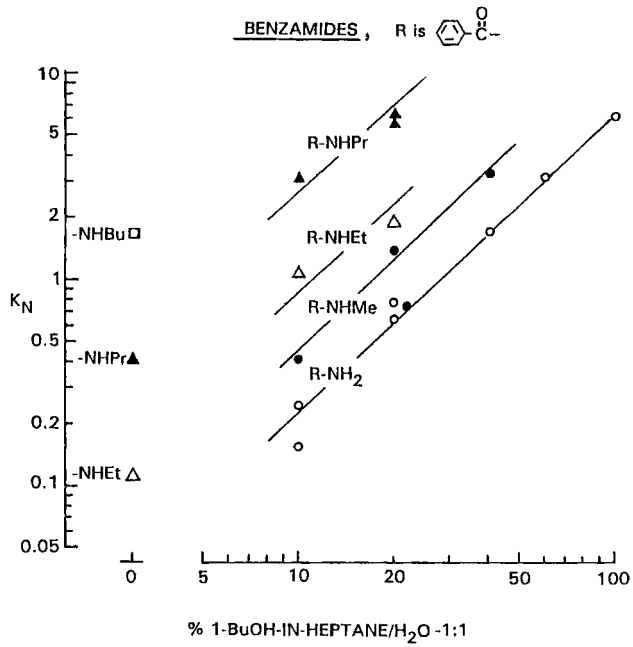

FIGURE 3. Relative polarity of heptane/1-butanol/water systems as indicated by partition coefficients for N-alkyl benzamides.

percentage of 1-butanol in heptane for 1:1 (v/v) systems. Addition of 10% 1-butanol to heptane increases the partition coefficient about 10-fold. Thereafter, successive doubling of the 1-butanol content increases K_N in roughly 2-fold increments.

Similar data for solutes from several chemical classes are summarized in Table 2. The effect of added 1-butanol increases as the hydrophilic character of the solute increases, as reflected by an increased hydroxyl content. Addition of small amounts of 1-butanol to heptane has only a modest effect, 1.3 to 2-fold, on K_N for ketones and N-alkylanilines and the effect remains about the same as the percentage is increased beyond 10%.

Table 2. Approximate increase in K_N with increasing 1-butanol content for heptane/1-BuOH/H_2O mixtures expressed as % 1-BuOH-in-heptane/H_2O - 1:1(v/v) systems.

Compound Type	0→10% 1-BuOH	Doubling 1-BuOH above 10%
PNP-Glucosides	*	5-fold
DNP - Amino Alcohols	*	4
Benzyl Alcohol	10-fold	3.5
N-Alkylbenzamides	10	2
Ketoalkanes	1.3	2
N-Alkylanilines	2	2

* insoluble in heptane

Separation of p-Nitrophenol Conjugates

Separation of p-nitrophenol and its glucuronide and sulfuric acid ester conjugates by reverse phase CCC in 1-butanol/bicarbonate buffer at pH 8.64 is shown in Fig. 4. The polymeric dye marker is slightly retained, as indicated by comparison of $t_{0,co}$ with $t_{0,0}$ but this has a relatively small effect on the calculated K_N values, which are listed in the figure for comparison. Decreasing the pH by 1.53 units to 7.15 increases the retention times significantly, the effect increasing with decreasing polarity of the solute, as shown in Fig. 5. When chromatographed under these same conditions, retention of the nonionizing glucose conjugate was not influenced by pH and it eluted with a K_N of 1.5 and 1.8 at pH 8.64 and 7.15 respectively. The small difference observed may result from differences in buffer ionic strength.

Separation of N-Alkylanilines

The variation of retention time and peak shape with mobile phase pH in heptane/aqueous buffer systems, for aniline and its N-methyl and N-ethyl

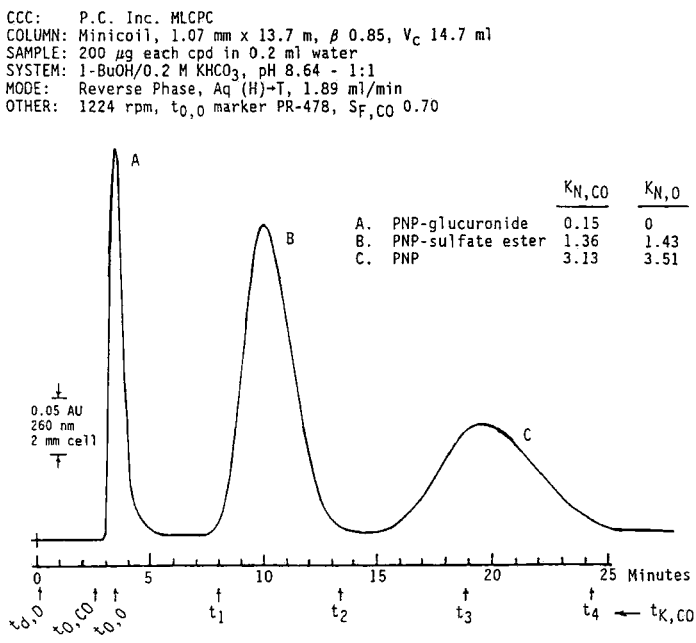

FIGURE 4. Separation of p-nitrophenol and its glucuronide and sulfuric acid ester conjugates by reverse phase CCC at pH 8.64.

derivatives, is summarized in Fig. 6. The values of K_N calculated from the chromatograms follow the expected curve for a plot of log K vs. pH as shown in Fig. 7. The pK_a values of 4.70, 4.67 and 5.23 for aniline and the N-methyl and N-ethyl derivatives obtained by least-squares regression analysis of the CCC data, are in reasonable agreement with the values of 4.58, 4.85 and 5.11 tabulated by Jencks and Regenstein.[7]

Symmetrical peak shapes are observed for the anilines at pH values as low as 3.4, almost two pH units below the pK_a of N-ethylaniline. However, at pH 2.76, the peaks for all three compounds are quite distorted. Distortion of peak shape was also observed for benzylamine and phenylethylamine and their N-

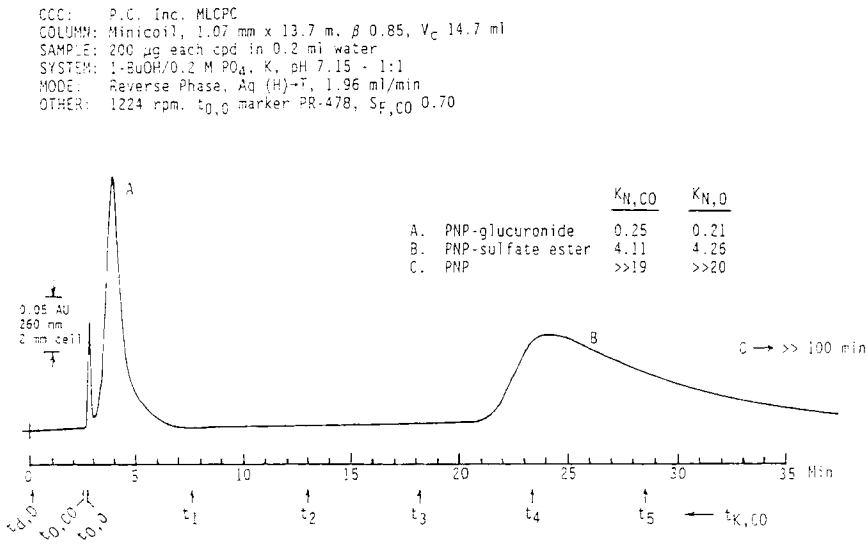

FIGURE 5. Separation of p-nitrophenol conjugates by reverse phase CCC at pH 7.15.

methyl derivatives, Fig. 8, even slightly above their pK_a values, although satisfactory chromatograms were obtained at pH values about 1.5 units or more above the pK_a. In the region where unsatisfactory chromatograms were obtained, the peak characteristically separated poorly from a nonretained portion of the same solute. This behavior is disappointing since it was hoped that the retention of lipophilic amine drug metabolites could be adjusted over a wide range at pH values below their pK_a, where higher chemical stability could be expected, and where their lower partition coefficients would provide shorter, more convenient retention times in reverse phase CCC.

The reason for poor chromatographic results at pH values below the pK_a is not known. The problem is not limited to the heptane/buffer systems, but was observed using heptane-1-butanol mixtures or neat 1-butanol as mobile phase. Poor aqueous solubility is an unlikely explanation since good performance is

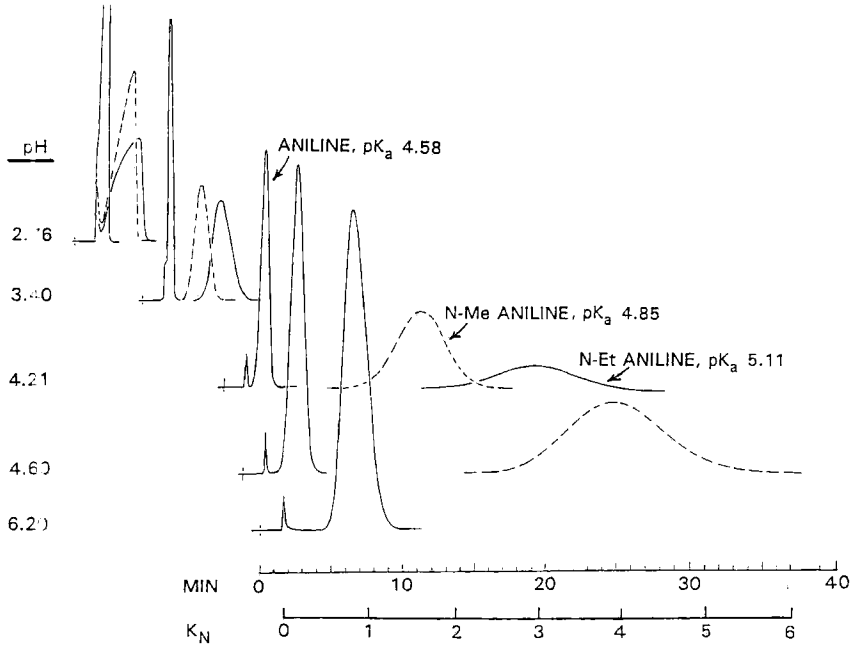

FIGURE 6. Effect of pH on peak shape and retention time for aniline, N-methylaniline and N-ethylaniline for reverse phase CCC in heptane/aqueous buffer. Flow rate is 2 ml/min. Buffers are 0.2 M acetic acid (pH 2.76), NH_4 formate (pH 3.40, 4.21, 4.60) and NH_4 acetate (pH 6.20). Samples are 0.2 ml of 10 μl amine/ml solutions in 0.2 M acetic acid. Marker for t_0 is DNPTK.

obtained at high pH, where the amines are least soluble in water. The injection of sample solutions more acidic than the mobile phase would cause a local shift in the pH of the 0.2 M mobile phase buffer, but tests with mobile phases having 0.6 and 1.2 M buffer concentrations did not improve the chromatogram. The results in Figs. 6 and 8 were obtained at a flow rate of 2 ml/min, but flow rates of 0.5 and 1 ml/min did not improve the chromatogram. Perhaps the problem arises from kinetic effects related either to ionization or hydration phenomena or to mass transfer of the nonionized species at the turbulent liquid-liquid interface.

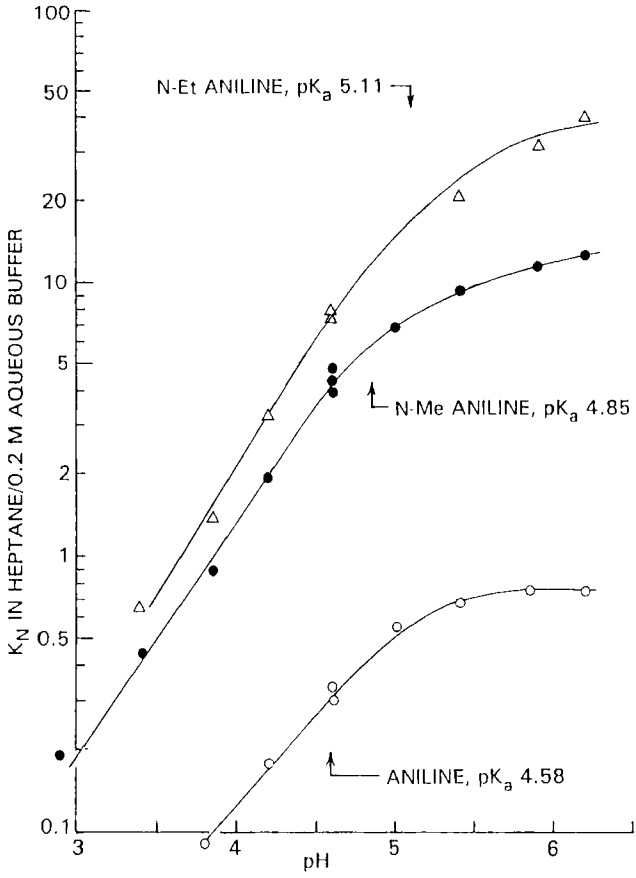

FIGURE 7. Semilog plots of K_N vs mobile phase pH for aniline, ○; N-methylaniline, ●; and N-ethylaniline, ▵. Arrow indicates position of published pK_a values.[7]

Separation of Other Amines

Countercurrent chromatography of amines more lipophilic than N-ethylaniline (K_N approx. 41, above the pK_a in heptane/water) is impractical at pH values above their pK_a, because of the long retention times encountered.

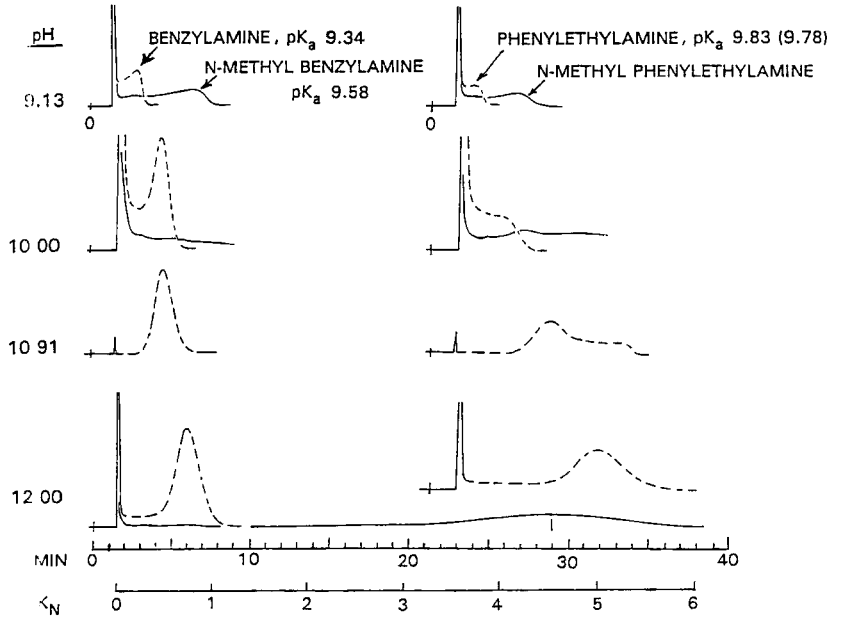

FIGURE 8. Effect of pH on peak shape and retention time for benzylamine and N-methylbenzylamine as well as phenylethylamine and N-methyl phenylethylamine for reverse phase CCC in heptane/aqueous buffer. Flow rate is 2 ml/min. Buffers are K phosphate (pH 9.13, 12.00), K carbonate (pH 10.00) and NH_3 (pH 10.91). Samples are 0.1 or 0.2 ml of 10 to 30 μl amine/ml in 0.2 M acetic acid. Marker for t_0 is DNPTK.

However, many aniline derivatives can be run successfully in the acidic range. For instance, N,N-dimethylaniline and N,N-diethylaniline have partition coefficients of 6.92 and 2.54 respectively at pH 3.80 as measured by reverse phase CCC in heptane/aqueous buffer. These values suggest the opposite order of lipophilicity expected from their structure. But the observed order results from the significantly higher pK_a of the diethyl derivative (pK_a = 6.56) compared with that for the dimethyl compound (pK_a = 5.06).[7] The plots of log K vs pH

(analogous to Fig. 7) overlap, causing a reversal in the order of chromatographic elution at pH values significantly below the pK_a.

The presence of an alcohol function, as might be encountered in drug metabolites, has a profound effect on retention. Whereas N-ethylaniline has a partition coefficient of about 41 at pH 6.18, with a resultant retention time of about 4 hrs. in the heptane/aqueous buffer system, 2-anilinoethanol is eluted at the mobile phase front (K=0). This large effect is due in part to the hydrophilic nature of the hydroxyl group and in part to an expected lowering of the pK_a by about one unit because of the proximity of the hydroxyl group to the nitrogen. A similar effect was seen in comparing the drug chloroquine, t_R 84 min, K = 14.3 with the drug hydroxychloroquine, t_R 5.1 min, K = 0.6 at pH 10. The hydroxyl group here is a 2-hydroxylethyl function, analogous to 2-anilinoethanol. Removal of an ethyl group to form desethylchloroquine or desethyl-hydroxyclororquine shifted the retention times to the mobile phase front (K=0).

Problems With Monitoring

Monitoring UV-absorbance produced very smooth baselines with reverse phase CCC with heptane/aqueous buffer systems. With 1-butanol/buffer systems, some noise was encountered as a result of droplet carryover and droplet adhesion to cell windows but it seldom led to serious interference. However, most ternary heptane/1-butanol/water systems produced very significant baseline noise, which made it difficult to monitor compounds having low absorptivity. Neither heating the effluent stream just prior to the detector to 50°C, nor attempts to bleed 2-propanol into the stream was successful in reducing the noise level.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the Northeast Regional Chromatography Discussion Group.

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