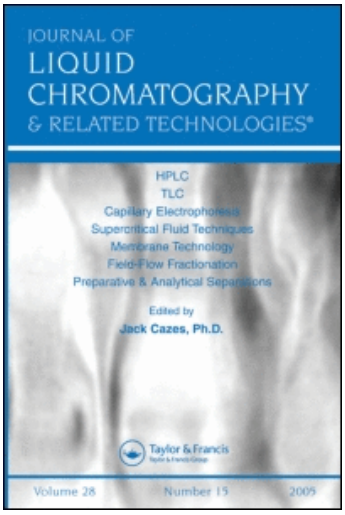

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USE OF TRIETHYLAMINE AS AN ION-PAIRING REAGENT

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ABSTRACT

Evidence that triethylamine (TEA) functions as an ion pairing reagent (IPR) in a mobile phase system (20 mmol/L acetic acid, 20 mmol/L phosphoric acid, 30 mmol/L TEA, pH 7.0) under both gradient and isocratic elutions is presented. The retention of anions (nicotinic, xanthurenic, anthranilic, and 3-hydroxyanthranilic acids) was increased with increasing TEA (cations) concentration; that of cations (benzylamine) was decreased and that of zwitterions or neutrals (kynurenine, 3-hydroxykynurenine, and aniline) exhibited no changes. TEA is shown to be a useful IPR in gradient elution due to its rapid equilibration time with the column. Proper selections of detection wavelength and gradient program are important to eliminate the interference of impurities in many commercial TEA preparations.

INTRODUCTION

Ion pair chromatography (IPC) is a powerful tool in the separation of ionic or ionogenic compounds (1-5). Although the actual mechanism involved in IPC is still not clear the phenomena seen are that the retention of compounds of the same charges are increased, while those of opposite charges are decreased and neutrals are not affected by increasing IPR concentrations within certain ranges (2,6,7). These phenomena make the concentration of the IPR an important parameter to control the retention and selectivity of ionic compounds in IPC (8-11).

Classified by their size, there are two types of IPR: small and large molecules (1,3). Large IPRs have one or more long hydrophobic tails (e.g. trioctylamine and heptanesulfonate). They are more strongly retained by the column, and are not easily flushed out of the column. Small counter ions (e.g. perchlorate, tetra-methylammonium) are more water soluble and are therefore easily eliminated from the column. Advantages of small IPRs are rapid column (re)equilibration time which makes them practical in gradient elution and the possibilities of mixed chromatographic behavior (1).

Triethylamine (TEA) is often added to mobile phases as a modifier or as a competing base to improve peak shapes and control the retention of amines (12-14). In addition, it has been used successfully as an IPR in the separations of nucleotides (15) and tryptophan metabolites in our laboratory (16) by reversed-phase liquid chromatography.

In the previous study (16), there are two points that may affect the relationship of retention and TEA concentration: 1) that the gradient elution may mask some retention changes of zwitterions (tryptophan, kynurenine and 3-hydroxykynurenine) when the TEA concentration is increased, and 2) the concentration of TEA was decreasing to some extent during the gradient elution as the TEA was not included in the acetonitrile reservoir. In addition, the effect of the TEA concentration on the retention of cations with increasing TEA concentration was not known since only anions and zwitterions were present in the mobile phase of the previous study (16).

This study is aimed to clarify these uncertainties and provide more evidences that TEA functions as an IPR in the optimized mobile phase system of the previous study (16). Aniline and benzylamine were included as model cation and neutral compounds respectively. The advantages and limitations of using TEA as an IPR in gradient elution are discussed.

MATERIALS

The HPLC system (Waters, Milford, MA, USA), reference standards and reagents used were as described in Chuang et al (16). Aniline (AN) and benzylamine (BA) were purchased from Aldrich Chemical Co. Chromatographic separation was performed on a Nova-Pak C_{18} steel column (150 mm x 3.9 mm i.d., 4 um).

METHODS

A) Gradient elution study

The chromatographic conditions were as described in previous study [16]. Duplicate runs of eleven reference standard mixture were analyzed by using two mobile phase systems. The first one is the optimized mobile phase developed in the previous study (16). This mobile phase (MP) consisted of a binary linear gradient of MP-A solution (20 mmol/L phosphoric acid, 20 mmol/L acetic acid, and 30 mmol/L TEA, pH 7.0 adjusted with 2 mol/L sodium hydroxide) and MP-B solution (acetonitrile). TEA (30 mmol/L) was included in MP-B acetonitrile reservoir in the second mobile phase system. The gradient program was started from 100%A to 20%A and 80%B within 20 minutes with a flow rate of 0.8 mL/minute.

B) Isocratic elution study

a) The effect of TEA concentration on the retention of anions and neutral compounds (Tryptophan metabolites of the kynurenine pathway):

The mobile phase was a 99:1 (v/v) mixture of buffer solution (20 mmol/L phosphoric acid, 20 mmol/L acetic acid, and 30 mmol/L TEA, adjusted to pH 7.0 with 2 mol/L NaOH) and acetonitrile. The column was equilibrated with a new mobile phase for at least 20 column volumes for each mobile phase change. The detector wavelength was 254 nm, with a sensitivity of 0.1 or 0.2 AUFS. Ten microliters of a six-reference mixture, containing kynurenine (KN), 3-hydroxykynurenine (HK), nicotinic (NA), xanthurenic (XA), anthranilic (AA)

and 3-hydroxyanthranilic (HA) acids were injected in duplicate and all chromatographic runs were performed at ambient temperature (22-24°C). The capacity factor (k') was calculated as follows: $k' = (k'/t_0)-1$, where t_0 is the column void time.

b) The effect of TEA concentration on the retention of cations and neutral compounds (Aniline and benzylamine):

The experimental conditions were as described above except that a buffer:acetonitrile mixture of 97:3 (v/v) was chosen as the mobile phase. The sensitivity was set at 0.010 or 0.050 AUFS. Ten microliters of AN and BA mixture (125 ng each) were injected for analysis.

RESULTS AND DISCUSSION

A) Gradient elution study

In order to keep the concentration of TEA constant during the gradient run, 30 mmol/L TEA was added to the reservoir containing acetonitrile (MP-B) for the gradient elution. In TABLE 1, the retention times of 11 compounds analyzed by two MP systems (with and without TEA added to MP-B) were shown and the difference in retention times for each compound was calculated.

The data shows that the differences, in the retention times of the eleven compounds, were negligible. This indicates that the effect of the inclusion of TEA in the acetonitrile reservoir on retention is minimal. This is probably due

TABLE 1. The effect of including TEA in MP-B (acetonitrile) on retention time

Compound	Retention time (min) Without TEA (A)	With TEA (B)	(B)-(A)
QA	3.01	3.03	+0.02
HK	3.65	3.62	-0.03
HA	4.69	4.63	-0.06
PA	5.97	5.95	-0.02
NA	6.70	6.67	-0.03
KN	7.74	7.66	-0.08
AA	10.85	10.95	+0.10
TRP	12.03	12.06	+0.03
XA	13.27	13.20	-0.07
KA	14.83	14.81	-0.02
QN	16.98	16.97	-0.01

The data shown above are average retention times of duplicate runs.

to the narrow gradient changes (0-15%) of acetonitrile (or TEA) during the chromatographic run.

B) Isocratic elution studies

a) The effect of TEA concentration on the retention of anions and neutral compounds:

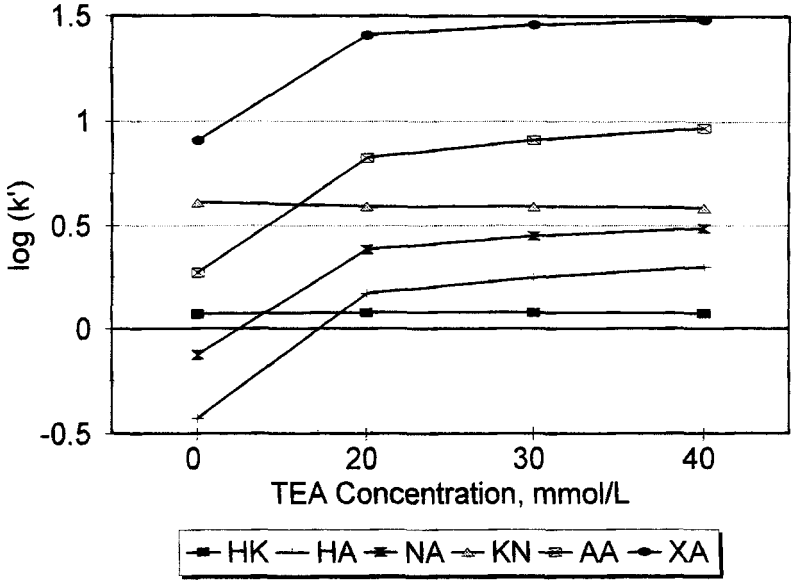

Figure 1. The effect of TEA concentration on the retention of six TRP metabolites. HK, 3-hydroxykynurenine; HA, 3-hydroxy- anthranilic acid; NA, nicotinic acid; KN, kynurenine; AA, anthranilic acid; XA, xanthurenic acid.

In FIGURE 1, the effect of TEA concentration on retention of the six compounds analyzed by isocratic elution is shown. The retention is expressed as the log(k') to show the curves of early eluted peaks clearly. The retention of four compounds (HA, NA, AA, and XA) increases along with the increase in TEA concentration, while those of HK and KN are not affected up to 40 mmol/L TEA, the highest concentration used in this study.

The isocratic study was performed to validate the findings of the gradient elution. The data shows no difference compared to the gradient analysis (16): the retention of anions (HA, AA, NA, and XA, containing opposite charged ions

vs TEA cation) is increased with TEA concentration increase and the retention of zwitterions (HK and KN) is not affected, indicating that TEA is acting like an IPR in this analytical system.

b) The effect of TEA concentration on the retention of cations and neutral compounds (Benzylamine and aniline):

Benzylamine (BN) and aniline (AN) were used as model cation and neutral compound in contrast to tryptophan metabolites which are anions or neutrals. The effect of the TEA concentration on the retention of AN and BN analyzed by isocratic elution is shown in FIGURE 2. The retention time of BN decreases when TEA is added to the mobile phase, and reaches a minimum at a TEA concentration of 10 mmol/L. However, the retention of AN is only slightly affected by changes in TEA concentration up to 40 mmol/L. The aromatic amine functional group of AN (pKa 4.6, ref. 17) is not in the ionized form under the conditions (pH 7.0) of the mobile phase, so that the retention of AN is not affected by TEA addition and TEA concentration changes. However, the aliphatic amine functional group of BN (pKa 9.35, ref. 18) is cationic under the same conditions, therefore the retention of BN is decreased by addition of cationic TEA ions.

These data show that under both gradient and isocratic elutions the retention of anions is increased with increasing TEA concentration; that of cations is decreased and that of zwitterions or neutrals, no changes, and thus support that TEA functions as an IPR under this mobile phase system.

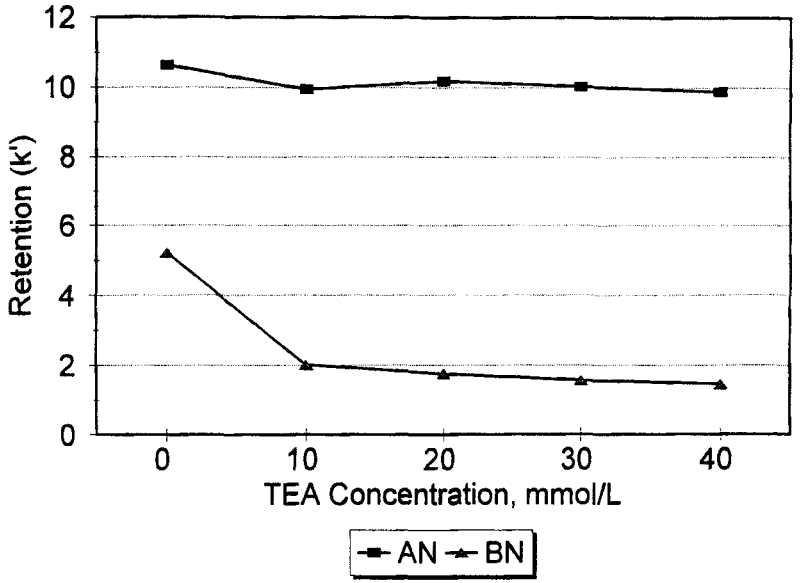

Figure 2. The effect of TEA concentration on the retention of aniline (AN) and benzylamine (BN).

It has been suggested that TEA does not have a deleterious effect on the stationary phase like that of tetrabutylammonium and is very water soluble and also has a low absorbance in the UV range (15). According to Gloor and Johnson's classification (1), TEA is a small IPR since it has three short hydrophobic alkyl chains. Therefore, TEA is not only rapidly (re)equilibrated in the column but is also easily eliminated from the column, making it practical for use in gradient elution.

From our experience with TEA, it equilibrates and re-equilibrates (during gradient elution) with the column very rapidly. However, it was found that

some TEA impurities were trapped at low acetonitrile concentrations (0-5%) in the gradient elution and then eluted at higher concentrations of acetonitrile, which perturbs the chromatographic baseline. We have tested on three brands of TEA preparation, all preparations have this problem. This problem is most apparent when a high sensitivity setting and a low wavelength (254 nm) were used in the detection. Most of these impurity peaks were not observed at a detection wavelength of 340 nm (19).

CONCLUSION

TEA functions as an IPR in an optimized mobile phase system on the observations that the retention of compounds with opposite charges (anions) was increased with TEA (cations) concentration increase; retention of compounds with same charges (cations) was decreased and that of neutrals (or zwitterions), no changes. In gradient elution TEA is a practical IPR due to its short (re)equilibration time. A precaution of using TEA in gradient is that many impurities are present in the commercial preparations. Proper selections of detection wavelength and gradient program are important to eliminate the interference of impurities.

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