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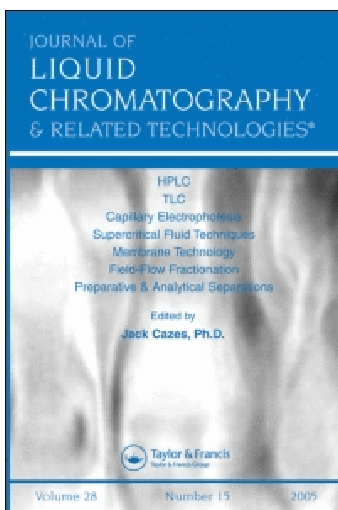

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Alkylamine/Phosphoric Acid as a Universal Buffer System for Basic and Acidic Mobile Phases in HPLC

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ALKYLAMINE/PHOSPHORIC ACID AS A UNIVERSAL BUFFER SYSTEM FOR BASIC AND ACIDIC MOBILE PHASES IN HPLC

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ABSTRACT

This paper describes the production of a universal buffer system which can be used for acidic, neutral and basic mobile phases in HPLC. The mixture of alkylamine and phosphoric acid diluted with water gives the possibility to carry out separations with basic buffers on reversed phase columns with a silica gel support. The organic bases (n-butylamine / triethylamine) are less aggressive against the silica gel than mineral bases. The influence of different pH-values upon the quality of the separations is tested with some selected basic and acidic compounds.
In addition to the tests with an ODS column we investigated the chromatographic behaviour (at different pH-values) of a new HPLC material (Polymer Gel) which is not based upon a silica gel support.

[1] BWA 430C from Wellcome Research Laboratories: Beckenham (Kent/UK) and Research Triangle Park (North Carolina/USA).

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INTRODUCTION

Lamotrigine[1] (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine) is a new anti-epileptic drug (AED) [1] which is tested in a clinical trial in the epilepsy center "Bethel" since 1986.

Lamotrigine (LAMO) is a basic and relatively polar compound. Basic substances can cause problems in the chromatographic analysis on reversed phase (RP) columns with acidic or neutral mobile phases. Therefore, the first method for the HPLC analysis of LAMO in human serum samples was developed by G. S. Land [2,3] using a silica gel column and an ammoniacal mixture of hexane/ethanol as mobile phase.

As chromatographic methods based on separations with silica gel columns are less flexible in practical use than those developed with RP-columns J.W.A. Mejer [2,3] analysed LAMO using an ODS-column (12.5 cm, 5 um particles) and a neutral mobile phase. With a sodium acetate buffer/acetonitrile mixture and a flow rate of 3 ml/min a retention time (RT) of 1.9 min for LAMO and of 3.1 min for carbamazepine (CBZ) was measured.

Under these chromatographic conditions some interfering endogenous compounds can be eluted in the same RT region as LAMO, so that some false positive results were obtained from the analysis of Lamo free samples.

Our own experiments with the separation of LAMO on an ODS-column using the acidic buffer system (pH 4.5) of

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the routine analysis of AEDs [4] showed a LAMO peak with a remarkable tailing. This effect is obviously caused by a reaction of LAMO with the residual silanol groups in the silica support of the RP-column.

The more polar a basic compound is the more this "secondary" effect competes with the hydrophobic interactions of the solute with the lipophilic surface of the stationary phase [5,7].

Some authors circumvent these problems in the chromatographic analysis of amines on RP-columns by adding triethylamine (TEA) or PIC-reagents (for "paired-ion chromatography") to acidic mobile phases [6-8], or by developing a new RP material especially designed for basic substances [9].

We started trials with basic buffer systems to achieve sharp peaks for LAMO which can be intgrated with sufficient precision. It is known, that anorganic buffers (e.g. phosphate or carbonate buffers) can solve the silica base of the RP-column and thereby shorten the column life time drastically.

Therefore, we replaced anorganic salts by organic bases (alkyl amines) expecting them to be less aggressive to silica gel than mineral bases. To avoid problems by the UV-cutoff of the mobile phase we took amines with short alkyl chains (n-butylamine or triethylamine) as organic bases for our investigation.

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MATERIALS AND METHODS

Apparatus and Reagents

The HPLC 1090 with an diodearray detector was obtained from Hewlett/Packard, Böblingen (FRG).
The analytical columns (200 mm, 2.1 mm ID) were filled with Shandon Hypersil(R) ODS (5 um) or Polymer Gel(R) from MZ-Analysentechnik, Mainz (FRG).

Acetonitrile ChromAR(R) was from Promochem, Wesel (FRG), water for HPLC from Baker Chemicals, Deventer (NL) and the other chemicals (analytical reagent-grade) from Merck, Darmstadt (FRG).

The common AEDs and metabolites named in table 2 were obtained from Promochem or from Aldrich, Steinheim (FRG). LAMO and DMT were from Wellcome, Hannover (FRG).

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Test Mixtures

LAMO, DMT, MONA, PI (full names see in Results and Discussion) and CBZ were dissolved in methanol (5 mg in 5 ml). Mixture 1 contained LAMO, DMT and MONA (1/1/10) whereas for mixture 2 methanol was added to PI and CBZ (7/2/1). For the tests with the "common" AEDs the stock solution for the production of calibration samples [10] was taken.

Buffer Solution pH 5

To 1000 ml of water 1 ml of a 10% phosphoric acid (in water, v/v) is added (pH ca. 3). After the addition of 1.6 ml of a 10% BUA (n-butylamine in water, v/v) the pH-

value of the mixture is ca. 4.5 and is adjusted with the BUA-solution to pH 5.

Buffer Solution pH 7

To 1000 ml of water 1 ml of the 10% phosphoric acid and 2.5 ml of the 10% BUA-solution are added. The pH is 7.0 using "Baker" water (otherwise the mixture is adjusted by adding dropwise one of both 10% solutions).

Buffer Solution pH 9

To 1000 ml of water 2 ml of the 10% BUA-soltuion and 0.5 ml of the 10% phosphoric acid are added (pH ca. 9.5). The mixture is adjusted to pH 9.0 by adding some drops of the 10% phosphoric acid.

Isocratic Separation Parameters

For the isocratic separations of test mixture 1 and 2 the buffer solutions and acetonitrile were mixed 70:30 (v/v) by the HPLC apparatus[2]. The flow was 0.3 ml/min. The detection wavelength was 205 nm, the column temperature 40°C and the injection volume 1 ul.

Gradient separation parameters

The separation of the AED stock solution started with %B (acetonitrile) = 17.5, and the gradient mixture was at min

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[2] The isocratic and the gradient mixtures of the buffer solutiones and acetonitrile in this paper were carried out by the HPLC apparatus to make sure that the percentage of acetoni- trile in all allied test series is always exactly the same.
For routine purposes it has to be recommended to produce pre- mixtures of buffer and acetonitrile (with a minimum of 10% acetonitrile) to avoid problems with degassing and storage [10].

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0.1 %B = 17.5, at min 9 %B = 30, at min 11 %B = 30 and at min 11.1 %B = 17.5. The flow was 0.3 ml/min, the column temperature was 65°C, the detection wavelength 205 nm and the injection volume was 3 ul.

RESULTS AND DISCUSSION

First of all, we developed a separation for LAMO and CBZ with a mixture of acetonitrile/methanol/phosphoric acid-TEA buffer (pH 8.5). As the internal standard (IS) from Wellcome (BWA 725C = 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine (DMT)) was eluted too early under these conditions we used a commercially available substance, 4-methoxy-2-nitroaniline (MONA), as internal standard for our investigation [2].

The positive experiences with the column lifetime using this basic buffer system encouraged us to replace also our common acid buffer by the phosphoric acid/alkyl-amine buffer system. One of the main advantages of both components added to mobile phases is the fact, that both are soluble in organic solvents as for instance acetonitrile or methanol.

Therefore, no micro precipitations of salt crystalles in the HPLC apparatus occur as they sometimes can be observed using phosphate buffers. The time before a blockage of in-line filter frits, microbore capillaries (0.12 mm ID) and columns can thereby remarkably prolonged. After about two years of practice with the two applica-

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tions mentioned above, we found the alkylamine/phosphoric buffer system to be so convenient that some aspects of it general use should be investigated and reported on.

We compared the influences of different pH values on selected examples of substances with amino groups (LAMO, DMT, MONA, CBZ and 2-phenylimidazole (PI)) by the evaluation of the corresponding retention shifts, measured as k'-values ($k' = \{t_R/t_0\}-1$). The RT of methanol was defined as t_0. The other chromatographic parameters: 1) analytical column, 2) column temperature, 3) flow rate, 4) content of acetonitrile remained in all tests the same.

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The effects of the three pH values of the mobile phases upon the separation results for the five selected substances were very different. LAMO, DMT and PI are strongly affected by a change from a basic to an acidic pH value. The k'-values were higher with the pH 5.0 buffer, but the peaks were very broad and DMT and LAMO were not separated anymore. MONA

TABLE 1

Different k'-values of the five test substances using an ODS-column with different pH-values of the buffers

substance	pH 9.0	pH 7.0	pH 5.0
k' of PI	0.86	0.89	2.24
CBZ	3.76	3.69	3.63
DMT	0.34	0.38	2.73
LAMO	0.98	0.99	2.73
MONA	3.23	3.18	3.19

t_0 (methanol) = 1.40 min

remained absolutely unaffected by a change from pH 9 to pH 5 whereas CBZ shows a very slight decrease of its k'-values.

Obviously the influence of the pH upon the separation parameters decreases the more the lipophilicity is the dominating part in the chemical charakter of the compound investigated. This could be the case with CBZ and MONA as their lipophilic interactions with the hydrophobic surface of the stationary phases are much stronger than the interactions of the primary amino groups with the unsaturated Si-OH groups in the column support.

The chromatographic results obtained using the pH 7-buffer were as good as those obtained at pH 9. A disadvantage of a neutral buffer by mixing an amine/acid combination might be, that the pH has to be precisely adjusted. One can avoid this problem by the use of an ammonium acetate buffer but, on the other hand, herewith the choice of the UV-wavelength for detection is restricted to higher values.

The alkylamine/phosphoric acid buffer can be used with low UV-wavelengths as they are, for instance, necessary for the analysis of AEDs [4,10].

A similar set of experiments as reported above (constant gradient separation of AEDs, only the pH values of the buffers were changed from pH 5 over pH 7 to pH 9) showed that also the AEDs and their metabolites are affected by the pH changes to very different extents.

As can be seen from table 2 the both compounds with an acidic charakter, phenobarbital (PB) and 5-ethyl-5-(p-tolyl) barbituric acid (ETB), show the greatest RT shifts.

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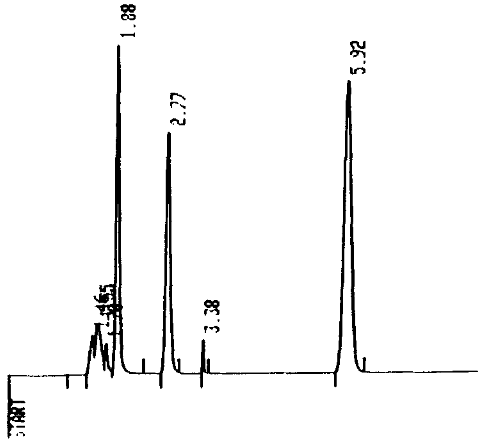

FIGURE 1 Isocratic separation of DMT (RT:1.88 min), LAMO (2.77) and MONA (5.92) at pH 9.

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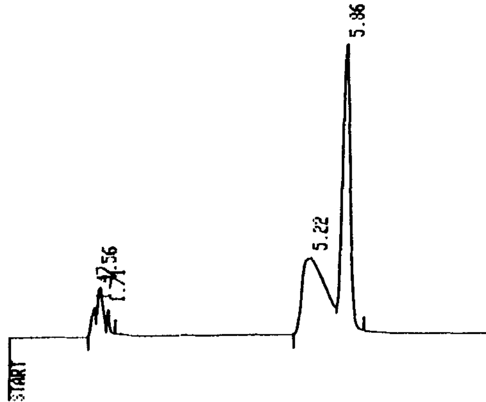

FIGURE 2 Isocratic separation of DMT (5.22), Lamo (5.22) and MONA (5.86) at pH 5.

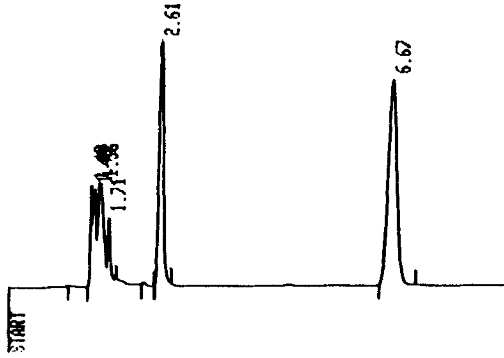

FIGURE 3 Isocratic separation of PI (2.61) and CBZ (6.67) at pH 9.

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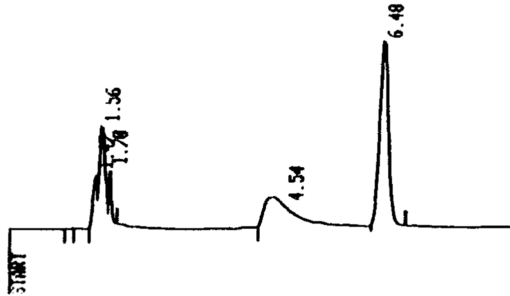

FIGURE 4 Isocratic separation of PI (4.54) and CBZ (6.48) at pH 5.

TABLE 2

Different k'-values of AEDs and metabolites using an ODS column with different pH-values of the buffers

substance	pH 5.0	pH 7.0	pH 9.0
k' of PEMA	0.87	0.89	0.89
ET	1.16	1.18	1.10
PRI	1.49	1.52	1.51
DIOL	2.06	2.17	2.13
MPS	2.69	2.74	2.64
PB	3.05	2.22	0.55
DM	3.48	3.50	3.08
EPO	3.95	4.04	4.07
ETB	5.08	4.19	1.65
PT	5.93	5.84	4.63
CBZ	6.32	6.48	6.50

t_0 (methanol) = 1.50 min

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The RTs of phenytoin (PT) and N-desmethyl methsuximide (DM) are affected by the change to pH 9 to a lower extent. CBZ, as already measured under isocratic conditions (see table 1), primidone (PRI) and their metabolites CBZ-epoxide (EPO), CBZ-diol (DIOL) and phenylethyl malonediamide (PEMA) as well as ethosuximide (ET) are nearly unaffected by the pH shifts.

From another point of view Roos and Lau-Cam [7] investigated the influence of triethylamine (TEA) upon the separation parameters in the analysis of drugs with weak basic charakters. They used an acidic mobile phase with acetic acid and varying contents of the alkylamine. The addition of TEA to the mobile phase improved peak shapes by working as an silanol-masking agent.

They found in their experiments only a little loss of retention, but, in addition to the reduction of peak tailing, TEA to be useful as an selectivity-enhancing agent.

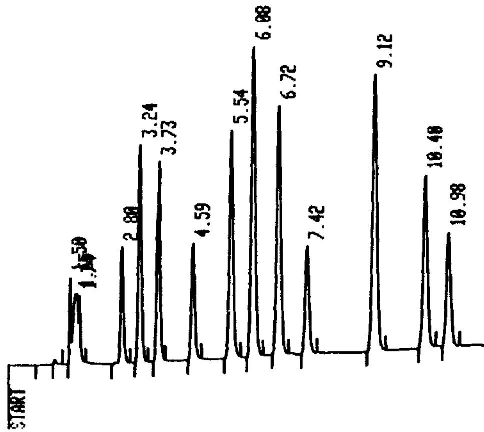

FIGURE 5 Gradient separation of AEDs at pH 5. PB (RT: 6.08) and ETB (9.12). For the RTs of the other AEDs see lit.[4].

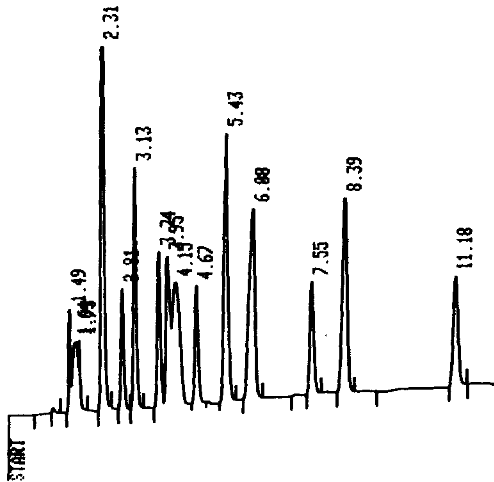

FIGURE 6 Gradient separation of AEDs at pH 9. PB (2.31) and ETB (3.95). The substance with the RT of 4.15 could be a decomposition product of ETB.

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Our own experiments with the cited [7] mobile phase with the same HPLC parameters used above (same column, flow, percentage of acetonitrile, temperature) showed a decrease of the k'-values for LAMO of ca. 12%, for DMT of ca. 9%, for MONA of ca. 7%, for PI of ca. 60%(!) and for CBZ of ca. 7% (k'-values are compared with those obtained by the isocratic separations using the mobile phase with pH 9.0 on the ODS column).

Compared with the buffers we reported on, the concentration of the alkylamine in the buffer solution in [7] is about 50 times higher. This could lead to an earlier blockage of the fluid system in microbore HPLC.

A second disadvantage is the high UV-cutoff of the acetic acid. A buffer system based on the components phosphoric acid and an alkylamine with a short alkyl chain is the most flexible in respect of the detection wavelength to be chosen.

Another possibility for HPLC analyses of basic or acidic compounds was recently developed by two groups of the University of Mainz[3] (FRG).

This new stationary phase is a polymer, based on a silica free support. Therefore the specifity of an HPLC column with this material differs from the ODS-column used in this paper. table 3 shows the greatest deviation for the k'-value of MONA, compared with the k'-values obtained by the separations on the ODS-column. The most interesting effect is that the k'-values

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[3] Polymer Service and MZ-Analysentechnik, Wöhlerstr. 2-6, D-6500 Mainz 1 (FRG).

TABLE 3

Different k'-values of the five test substance using an Polymergel column with different pH-values of the buffers

substances		pH 9.0	pH 7.0	pH 5.0
k' of	PI	1.06	1.06	1.04
	CBZ	4.06	4.11	4.04
	DMT	0.28	0.31	0.48
	LAMO	0.94	0.99	1.15
	MONA	7.98	7.98	7.99

t_0 (methanol) = 1.60 min

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of the separations carried out with the Polymer Gel(R) column are not depending on the pH-value of the mobile phase.

This is an indirect proof of the assumption that the effects upon the amines by separations on ODS material are due to side-reactions with the unsaturated silanol groups of the column support.

CONCLUSIONS

Our experiences during about two years of practice in using alkylamine/phosphoric acid as buffer system for mobile phases in HPLC show that both components give an ideal combination for the following reasons:

1) Both components are soluble in the organic solvent acetonitrile. Therefore any ratio of solvent and aqueous buffer can be produced without the precipitation of salts if the content of the organic solvent gets higher than 75%.

2) Alkylamines with short alkyl chains and phosphoric acid have both low UV-cutoffs and are therefore suitable for gradient elutions with low detection wavelengths.

3) For separations using a mobile phase with a basic pH the organic bases are less aggressive to the silica support of reversed-phase columns than anorganic salts. Using the Hypersil(R) ODS column, the pH of the mobile phases can be changed several times before the separation quality decreases.

A suitable alternative to the use of a basic buffer system for separations of basic compounds on an ODS-column can be the use of the Polymer Gel(R) column, with which the separations are not depending on the pH of the mobile phase.

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