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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SOME INDOLE AND QUINOLINE ALKALOIDS FROM *CINCHONA*

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SUMMARY

A high-performance liquid chromatographic method was developed for the separation of corynantheal, quinamine, cinchonamine, cinchonine, cinchonidine, quinidine and quinine. The eluent consists of 0.1 *M* potassium phosphate buffer (pH 3.0)–acetonitrile (85:15) to which is subsequently added 5 m*M* hexylamine, 85% phosphoric acid to give pH 3.0 and 2% (v/v) tetrahydrofuran. It proved necessary to develop another reversed-phase eluent, namely 1 *M* sodium acetate buffer–acetonitrile–tetrahydrofuran (80:20:5) (pH 5.5–6.0) for the separation of cinchoninone, quinidinone, cinchonine, cinchonidine, quinine and quinidine because of the epimerization of cinchoninone and quinidinone at one of the asymmetric carbon atoms during the chromatographic run. Both eluents were used in combination with a LiChrosorb RP-8 Select B column.

INTRODUCTION

The importance of and demand for the products of the *Cinchona* tree, *viz.* quinine (Q) and quinidine (Qd), as well as the non-methoxylated analogues cinchonidine (Cd) and cinchonine (C), is well known. The production of these quinoline alkaloids by means of plant cell cultures is under investigation in our laboratory. The fundamental knowledge of the biosynthesis of the *Cinchona* alkaloids in plant cell cultures still displays several gaps. Corynantheal (CorAL) is thought to be a key product in the biosynthetic pathway.

To investigate its conversion into the quinolines and to determine the activities of the enzymes involved, a high-performance liquid chromatographic (HPLC) system suitable for the separation of CorAL, cinchonamine (CAm), quinamine (QAm), Cd, C, Q and Qd had to be developed (see Fig. 1). As we also wanted to elucidate the enzymatic reaction of cinchoni(di)none (C-on) into C and Cd and of quini(di)none (Q-on) into Q and Qd, a system for the separation of these compounds was also required. It would be convenient to have one system for both assays. Therefore, the emphasis here was to analyse the end-products of the biosynthesis pathway in addition to the intermediates leading to these end-products.

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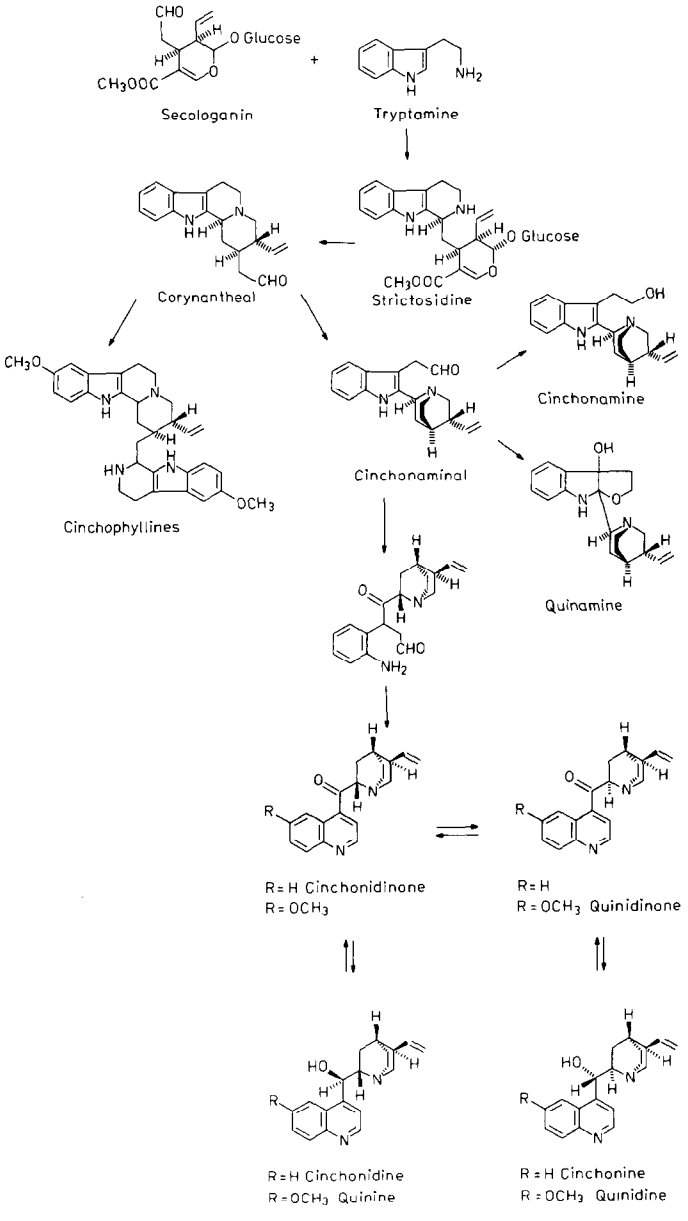

Fig. 1. Biosynthesis of *Cinchona* alkaloids.

So far some work has been done on the HPLC separation of *Cinchona* alkaloids[1]. However, in none of these systems was attention paid to the separation of the indole alkaloids CorAL, QAm and CAm in combination with the quinoline alkaloids.

In view of the procedures used to extract the compounds of interest of the

biomass, a reversed-phase (RP) HPLC system was to be preferred, with dihydroquinine (HQ) as the internal standard. HQ is preferred because its extraction properties are the same as those of the alkaloids to be separated. Strong bases, such as the quinoline alkaloids, usually show poor peak shapes in the reversed-phase HPLC mode. Hexylamine, as a long-chain molecule with an alkaline end-group, can be added to the eluent to improve the shape of the peaks of the eluted alkaloids, owing to its capacity to mask the free silanol groups of the column material[2].

EXPERIMENTAL

Apparatus

The HPLC system consisted of a high-pressure pump (Model 2150; LKB, Bromma, Sweden), a 100-μl loop injector (U6K; Waters Assoc., Milford, MA, U.S.A.), a 275-nm UV detector (2158 Uvicord SD; LKB) and a flat-bed recorder (BD41; Kipp, Delft, The Netherlands). A 25 cm × 4 mm I.D. RP-8 Select B column of LiChrosorb (5 μm) (Merck, Darmstadt, F.R.G.) was used. The flow-rate was adjusted to 1.5 ml/min for all analyses.

Alkaloids

Cinchonine, cinchonidine, quinine and quinidine were obtained from Fluka (Buchs, Switzerland) and cinchoninone and quinidinone from ACF Chemiefarma (Maarssen, The Netherlands). Quinamine and cinchonamine were obtained from plant material and corynantheal by semisynthesis by Dr. G. Massiot (Laboratoire de Pharmacognosie, Faculté de Pharmacie, Reims, France).

Chemicals

Analytical-reagent grade materials were used unless indicated otherwise and all water used was deionized and filtered.

Acetonitrile and methanol were freshly distilled before use. Tetrahydrofuran was of extra-pure quality (Merck), 1,4-dioxane and acetic acid were of Baker grade (Baker, Deventer, The Netherlands), phosphoric acid was 85% (Merck), hexylamine was of "for synthesis' quality (Merck) and KH_2PO_4 was obtained from Baker.

Eluents

The eluent described by McCalley[3] and used as a basis for developing a new system was prepared as follows: 0.1 *M* KH_2PO_4 solution was acidified with 0.1 *M* H_3PO_4 to pH 3.0 and 850 ml of this buffer were mixed with 150 ml of acetonitrile. Eluent A was prepared from this solution by the addition of 5 m*M* hexylamine, acidification to pH 3.0 with 85% H_3PO_4 and subsequent addition of 2% (v/v) tetrahydrofuran. The eluents were filtered and degassed before use.

RESULTS AND DISCUSSION

McCalley[3] compared the performances of six different RP columns related to the separation of the major *Cinchona* alkaloids; two of these (μBondapak C_{18} and LiChrosorb RP-8 Select B) gave good results without the use of a long-chain amine. We chose the LiChrosorb RP-8 Select B column and the eluent he described as a starting point for the development of a suitable system for our assays.

This system gave a poor separation of Qam, Q and CorAL and a very broad peak for CorAL. Moreover, C-on and Q-on both displayed two peaks, a small sharp one and a larger, very broad one (see Fig. 5 and Tables I–IV, where the first peak is indicated in parentheses). This phenomenon will be discussed below.

For optimization of the system, changes in the selectivity (pH, addition of hexylamine, addition of modifiers such as tetrahydrofuran and dioxane) of the eluent have been studied. An acceptable separation for most of the compounds was achieved; only the analysis of Q-on and C-on failed with this system. A separate RP eluent was developed for the separation of these ketones, C, Cd, Q, Qd and HQ, as described below.

Optimization for all components, except C-on and Q-on

Influence of pH. The pH was varied between 3.0 and 5.7. An increase in the pH caused increased retentions of C, Cd, Q, Qd and HQ. Differences in pH had less influence on the capacity factor, (k') of the weakly basic compounds CorAL, QAm and CAm. The shape of the corynantheal peak improved at lower pH (see Table I and Fig. 2). By varying the pH, the separation between the quinoline alkaloids and the indole alkaloids could be improved; however, within these groups little influence of the pH on the selectivity of the system was observed.

Influence of hexylamine. The addition of 5 m*M* hexylamine reduced the k' values of all compounds. For C, Cd, Q, Qd and HQ no improvement in the separation was achieved; the shape of the CorAL and CAm peaks improved. Apparently hexylamine does not change the selectivity of the system. The effect of the addition is shown in Table II and Fig. 3. The concentration of hexylamine in the eluent should be kept low, in order to prevent crystallization of the eluent at the HPLC connections and in the autosampler. Therefore, a 10 m*M* hexylamine concentration was not tested; the 5 m*M* solution gave satisfactory improvements.

Influence of tetrahydrofuran (THF). The percentage of THF added, was varied, 0, 2 or 4% (v/v) being added. All components of the test mixtures had shorter retention times and all peak shapes improved on addition of THF. On the other hand, C

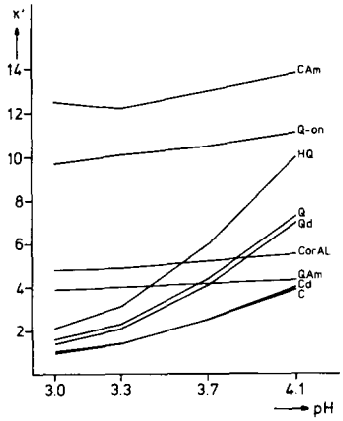

Fig. 2. Effect of pH on the capacity factors, k', of some quinoline alkaloids (C, Cd, Q, Qd, HQ and Q-on) and some indole alkaloids (CorAl, QAm, and CAm). For eluents, see Table I; eluents 3, 4, 5 and 6.

TABLE I

INFLUENCE OF pH

pH adjusted by means of H_3PO_4 or NaOH. Initial eluent: 0.1 *M* potassium phosphate buffer (pH 3.0)–acetonitrile (85:15).

No.	*Eluent*	*Plate number*		*k'*									
		HQ	*CorAl*	*HQ*	*CorAl*	*C*	*Cd*	*Q*	*Qd*	*QAm*	*CAm*	*C-on*	*Q-on*
1	Initial eluent + 5 m*M* hexylamine, pH = 4.06	3332	159	19.3	8.3	7.0	7.1	13.6	12.9	6.5	23.9	13.0	26.1
2	As 1, pH = 3.0	2800	323	4.1	7.3	1.5	1.7	2.9	2.6	5.7	21.6	11.1 (7.1)	21.6 (15.1)
3	Initial eluent + 5 m*M* hexylamine + 2% THF, pH = 3.0	4268	298	2.1	4.8	0.95	0.98	1.6	1.4	3.9	12.5	5.4 (3.4)	9.7 (6.4)
4	As 3, pH = 3.3	2652	371	3.1	4.9	1.4	1.4	2.3	2.1	4.0	12.2	5.7	10.1
5	As 3, pH = 3.7	2743	194	6.0	5.2	2.5	2.5	4.4	4.1	4.2	13.0	6.2	10.5
6	As 3, pH = 4.1	2100	174	10.0	5.5	3.9	4.0	7.3	7.0	4.3	13.8	6.3	11.1
7	Initial eluent + 4% THF, pH = 3.0	2064	233	1.5	4.9	0.74	0.70	1.2	1.1	3.8	11.5	4.3	7.4
8	As 7, pH = 3.5	1973	163	3.3	5.1	1.5	1.5	2.5	2.3	3.9	12.0	4.7	8.2
9	As 7, pH = 4.0	1940	–	6.4	5.5	2.7	2.7	4.8	4.6	4.2	12.9	5.2 (3.4)	9.1 (6.3)
10	Initial eluent + 5 m*M* hexylamine + 4% THF, pH = 4.0	1608	–	5.1	3.9	2.2	2.2	3.8	3.8	3.0	8.9	4.0	6.9 (fronting)
11	As 10, pH = 5.7	1382	–	10.9	–	4.6	4.3	8.2	8.5	3.6	10.9	8.1	13.3

TABLE II

INFLUENCE OF HEXYLAMINE

Initial eluent: 0.1 *M* potassium phosphate buffer (pH 3.0)–acetonitrile (85:15).

No.	*Eluent*	*Plate number*		*k'*								
		HQ	*CorAl*	*HQ*	*CorAl*	*C*	*Cd*	*Q*	*Qd*	*QAm*	*CAm*	*C-on*	*Q-on*
1	Initial eluent + 4% THF, pH = 4.0	1940	–	6.4	5.5	2.7	2.7	4.8	4.6	4.2	12.9	5.2 (3.4)	9.1 (6.3)
2	As 1, + 5 m*M* hexylamine, pH = 4.0	1608	–	5.1	3.9	2.2	2.2	3.8	3.8	3.0	8.9	4.0 (fronting)	6.9
3	Initial eluent, pH = 3.0	1357	245	4.3	10.3	1.6	1.7	3.1	2.8	–	30.2	14.0 (9.3)	27.4 (19.0)
4	As 3, + 5 m*M* hexylamine	2800	323	4.1	7.3	1.5	1.7	2.9	2.6	5.7	21.6	11.1 (7.4)	21.6 (15.1)

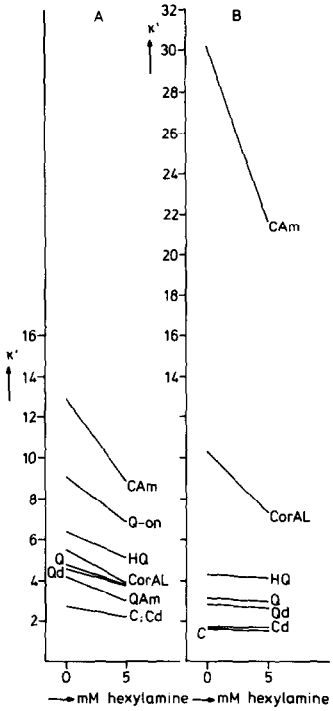

Fig. 3. Influence of hexylamine on the retention of quinoline alkaloids (C, Cd, Q, Qd, HQ, Q-on) and indole alkaloids (CorAL, QAm, CAm). For eluents, see Table II; for A eluents 1 and 2 and for B eluents 3 and 4.

and Cd now had almost identical k' values, and also for Q and Qd there was a tendency to co-elute, especially at the 4% THF level. The peak shapes of CorAL and CAm improved and the shorter analysis time, acquired by the lower k' of the last compound, CAm (17 *vs* 28.5 min), was an additional advantage. Results are given in Table III.

Influence of dioxane and pyridine. Modifiers other than THF were studied. Addition of 1,4-dioxane (2%, v/v) to the eluent resulted in only a slight improvement in peak shape. Retention times were shortened, but overall THF gave better results. Addition of pyridine to the eluent (1 m*M* pyridine; the pH was readjusted to 3.37 with H_3PO_4) did not improve the performance. Results are given in Table IV.

From these results, an eluent containing 5 m*M* hexylamine and 2% (v/v) THF at pH 3.0 was selected as the most suitable for the assay of CorAL, QAm, CAm, C, Cd, Q and Qd (eluent A; see Fig. 4). A disadvantage is that eluents containing hexylamine crystallize very easily at the HPLC connections, causing problems, especially in autosamplers. Nevertheless, the advantage of better peak shapes prevailed over this inconvenience.

Optimization for all quinoline alkaloids (C-on, Q-on, C, Cd, Q, Qd, HQ)

Eluent A was not suitable for the assay of the ketones (Q-on and C-on). Both Q-on and C-on gave two peaks in this solvent (see Fig. 5; in Tables I–IV the first of

TABLE III

INFLUENCE OF THF

Initial eluent: 0.1 *M* potassium phosphate buffer (pH 3.0)–acetonitrile (85:15).

No.	*Eluent*	*Plate number*		*k'*								
		HQ	*CorAl*	*HQ*	*CorAl*	*C*	*Cd*	*Q*	*Qd*	*QAm*	*CAm*	*C-on*	*Q-on*
1	Initial eluent + 5 m*M* hexylamine, pH = 3.0	2800	323	4.1	7.3	1.5	1.7	2.9	2.6	5.7	21.6	11.1 (7.4)	21.6 (15.1)
2	As 1, + 2% THF	4268	298	2.1	4.8	0.95	0.98	1.6	1.4	3.9	12.5	5.4 (3.4)	9.7 (6.4)
3	Initial eluent, pH = 3.0	1357	245	4.3	10.3	1.6	1.7	3.1	2.8	–	30.2	14.0 (9.3)	27.4 (19.0)
4	As 3, + 2% THF	1326	236	2.5	6.9	1.1	1.1	1.8	1.7	–	18.1	7.4 (4.8)	13.5 (9.1)
5	As 3, + 4% THF	2064	233	1.5	4.9	0.74	0.70	1.2	1.1	3.8	11.5	4.3 (2.8)	7.4 (5.0)

TABLE IV

INFLUENCE OF DIOXANE AND PYRIDINE

Initial eluent: 0.1 *M* potassium phosphate buffer (pH 3.0)–acetonitrile (85:15).

No.	*Eluent*	*Plate number*		*k'*									
		HQ	*CorAL*	*HQ*	*CorAL*	*C*	*Cd*	*Q*	*Qd*	*QAm*	*CAm*	*C-on*	*Q-on*
1	Initial eluent, pH = 3.3	1584	195	10.1	10.7	3.8	4.0	7.3	6.6	–	–	17.0 (fronting)	–
2	As 1, + 2% dioxane, pH = 3.38	1825	197	7.5	8.2	2.9	3.0	5.4	4.9	6.7	22.8	12.0 (much fronting)	22.6
3	As 2, + 1 m*M* pyridine pH = 3.37	1314	176	7.7	8.3	2.9	3.2	5.6	5.0	6.9	23.2	12.0 (much fronting)	23.0

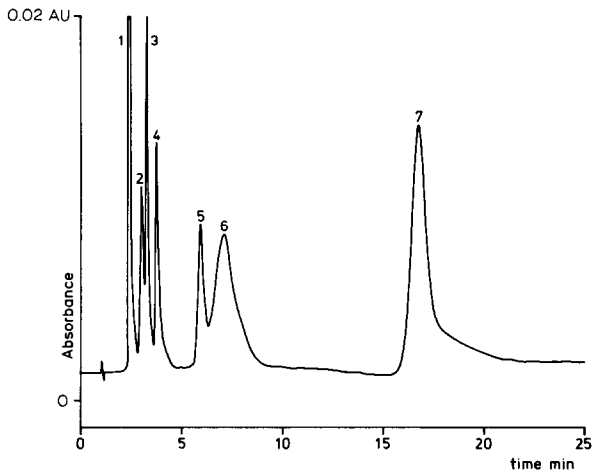

Fig. 4. Chromatogram of a test mixture. Peaks: 1 = cinchonine and cinchonidine (1.5 μg of each); 2 = quinidine (3 μg); 3 = quinine (3 μg); 4 = dihydroquinine (3 μg); 5 = quinamine (8 μg); 6 = corynantheal (2 μg); 7 = cinchonamine (4 μg). Eluent: 0.1 *M* potassium phosphate buffer (pH 3.0)–acetonitrile (85:15) plus 5 m*M* hexylamine; acidification to pH 3.0 with 85% H_3PO_4 and subsequent addition of 2% (v/v) tetrahydrofuran (eluent A). Column: LiChrosorb RP-8 Select B (25 cm); flow-rate, 1.5 ml/min.

the two peaks is indicated in parentheses). This can be explained by epimerization at C-8–C-9 (see Fig. 6), resulting in compounds with different stereochemistry, and consequently different chromatographic behaviour[4]. According to Robins and Rhodes[4], there is a slow mutarotation in polar solvents, resulting in a mixture of *R*- and *S*-epimers. The equilibrium of this epimerization proves to be pH dependent; at pH 4.5 or higher, of each of the ketones only one peak is visible in the chromatograms. Therefore, a rather high pH is needed for the analysis of the ketones. However, the other quinoline alkaloids (Q, Qd, C, Cd) showed poor peak shapes and very long retentions under such conditions, owing to the lower degree of protonation of the

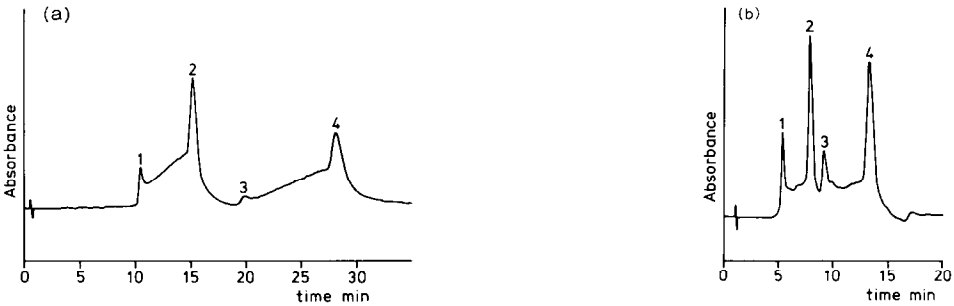

Fig. 5. Typical chromatograms of cinchoninone (1) and cinchonidinone (2), quininone (3) and quinidinone (4). (a) Eluent, 0.1 *M* KH_2PO_4 + 5 m*M* hexylamine in water–acetonitrite (85:15); H_3PO_4 added to pH 3.0 (eluent 1 in Table III). (b) Eluent as in (a) plus 2% (v/v) THF (eluent 2 in Table III). Flow-rate, amounts injected and detection are the same in both chromatograms.

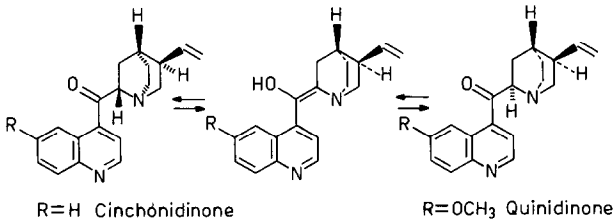

Fig. 6. Epimerization of C-on and Q-on.

alkaloids at higher pH. As is known from previous investigations[5], alkaloids will form ion pairs when acetate is the counter ion. In an HPLC eluent based on acetate buffer strong ion-pair formation is observed, even at higher pH; in phosphate buffer the alkaloids do not give rise to ion pairs extractable with non-polar solvents[5], *i.e.*, they will elute more rapidly than the alkaloid–acetate ion pairs. For acetate-based solvents a much higher concentration of acetonitrile is needed to ensure reasonable retention of the now less polar compounds.

At this time only the ketones were tested together with C, Cd, Q, Qd and HQ; as we already developed an HPLC system for the separation of CorAL, QAm and CAm in combination with these five alkaloids, their chromatographic behaviour in eluents based on an acetate buffer was not investigated.

Several acetate buffer eluents were tested (see Tables V and VI). The first was a 0.1 *M* acetate buffer (pH 5.5) mixed with an equal volume of methanol. The pH of this eluent after the addition of methanol was 6.4. The performance in this eluent was poor, with no separation between C and Cd or between Q and Qd, and severe tailing of all compounds. By varying the chromatographic parameters, attempts were made to find the optimum in separation quality. When methanol was replaced with acetonitrile as the organic part of the eluent in comparable volume ratios, the k' values decreased considerably owing to the higher eluotropic strength of acetonitrile (eluents with the same eluotropic strength were not compared). It is noteworthy that in acetate buffer–acetonitrile the sequence of elution is Cd–C–Q–Qd–HQ, whereas with methanol the sequence is C–Cd–Qd–Q–HQ (the same inversion in the retention of the stereoisomers was noted when phosphate buffer was used instead of acetate buffer).

Another variation was the acidity of the eluent: the pH was varied between 4.1 and 6.7, by adding 10 *M* sodium hydroxide solution or acetic acid; in the latter

TABLE V

ACETATE BUFFER–METHANOL ELUENTS

Eluent	k'						
	C	*Cd*	*Q*	*Qd*	*HQ*	*C-on*	*Q-on*
0.1 *M* sodium acetate (pH 5.5) + methanol (1:1), pH = 6.4	5.5	5.5	9.0	7.8	11.1	12.3	–
As above + acetic acid to pH 5.5	3.8	4.0	6.7	5.8	8.8	5.2	8.9

TABLE VI

ACETATE BUFFER–ACETONITRILE ELUENTS

Eluent		*k'*						
No.	*Composition*	*C*	*Cd*	*Q*	*Qd*	*HQ*	*C-on*	*Q-on*
1	0.1 *M* acetate (pH 5.5) + CH_3CN (1:1), pH = 6.7	2.1	1.9	2.2	2.4	2.7	3.9	4.9
2	1 + acetic acid to pH 5.5	1.1	1.1	1.3	1.4	1.6	1.7	2.2
3	2 + water to H_2O–CH_3CN (2:1), pH 5.05	2.5	2.3	3.2	3.5	4.0	3.2	4.5
4	3 + water to H_2O–CH_3CN (3:1), NaOH to pH 5.5	6.1	5.8	8.7	9.7	11.7	8.6	13.9
5	4 + acetic acid to pH 5.05	5.8	5.3	8.7	9.3	11.4	7.4	12.5
6	5 + 1% (v/v) THF	4.7	4.4	7.0	7.4	9.5	5.8	9.5
7	6 + water to H_2O–CH_3CN (4:1), pH 4.9	9.6	9.1	15.8	16.3	22.0	12.1	–
8	7 + acetic acid to pH 4.1	5.8	5.8	9.7	9.7	13.6	9.1	16.0
9	8 + THF to 5% (v/v), pH 4.15	2.5	2.5	3.7	3.7	4.8	3.2	5.1
10	9 + NaOH to pH 4.9	3.3	3.1	5.1	5.2	6.4	3.9	6.3
11	10 + NaOH to pH 5.5	4.1	3.7	6.0	6.5	7.8	5.4	8.4
12	11 + NaOH to pH 6.0	4.6	4.1	6.7	7.1	8.7	7.3	11.2

instance not only the [HA]:[Ac^-] ratio changed, but also the sum. In all instances a lower pH coincided with lower *k'* values.

The water to organic solvent ratio was varied between 1:1 and 4:1. The more water the eluent contained, the more all the compounds were retained. For example, in an eluent containing acetate buffer and acetonitrile, with 1% (v/v) THF added and a final pH of 5.0, the *k'* values of all compounds tested increased by a factor of 2 or more when the water to acetonitrile ratio was changed from 3:1 to 4:1.

The addition of THF to the eluent resulted in an improvement in the elution pattern; the peaks shapes were better and the retention times shorter. However, as in the phosphate buffer eluents, here also C and Cd had the same *k'* values and Q and Qd showed a tendency to co-elute when 5% THF was present. An increase in the THF content from 1 to 5% (v/v) in an eluent containing acetate buffer–acetonitrile (4:1) at pH 4.1 resulted in a 2–3-fold decrease in *k'* values. The same occurred with an eluent of pH 4.9.

Instead of THF, other organic modifiers from diverse classes in Snyder's classification[6] were tested. Both 1 and 5% (v/v) additions to the eluent of methoxyethanol, 1,4-dioxane and acetone were tried, but none gave satisfactory results; addition of chloroform, mixed with methanol to increase its solubility, gave no improvement.

When the column was situated in a water-jacket at 35°C, only a slight improvement in the separation was observed.

In spite of all these variations, no eluent could be found that fulfilled satisfactorily all the requirements stated above. The best eluent proved to be 1 *M* acetate buffer–acetonitrile (80:20) with pH between 5.5 and 6.0, to which 5% (v/v) THF was added. With this system C-on can be determined in the presence of C and Cd. However, C-on cannot be determined in combination with both Q *and* Qd; at pH 5.5 it elutes shortly before Q, without sufficient separation, and at pH 6.0 it elutes shortly after Qd.

For long-term satisfactory HPLC performance a lower molarity of the eluent is desired.

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