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ANALYSIS OF β-CARBOLINES BY REVERSED-PHASE ION-PAIR PARTITION CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic system was developed for the analysis of ten selected β-carbolines. It consists of tri-*n*-butyl phosphate as liquid stationary phase and buffered mixtures of water and methanol with perchlorate as the mobile phases. The separation was optimized by means of mobile phase parameters such as the perchlorate concentration, the methanol content and the pH. The relationship between these parameters and the retention of the β-carbolines was determined and compared with theory as derived from a model of the retention mechanism. The potential of spectrofluorometric and UV spectroscopic detection with regard to the detection limits, linear dynamic range and selectivity were investigated.

INTRODUCTION

β-Carbolines are the products of condensation of β-indole-3-ethylamines (tryptamine, serotonin and 5-methoxytryptamine) or β-indole-3-ethylamino acids (tryptophan) and an aldehyde (acetaldehyde, formaldehyde), see Fig. 1. In recent years a number of β-carbolines has been found to be present in humans and animals[1–7] at concentrations ranging from 1 ng/ml in blood, urine and cerebrospinal fluid to 10 ng/g in brain tissue. β-Carbolines can influence the activity of a number of enzymes, *e.g.*, monoamine oxidase (MAO)[8,9], and the uptake and release of some neurotransmitters in the central nervous system, *viz.*, serotonin, dopamine and noradrenaline[9–11].

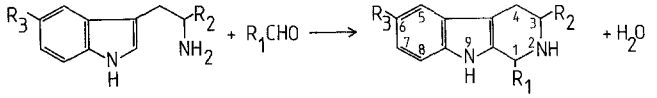

Fig. 1. Formation of β-carbolines.

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They can also interact with a number of receptors, *e.g.*, the benzodiazepine receptor[12]. The role of β-carbolines *in vivo* is still unknown. However, attention has been focused on a possible rôle in psychic disorders, schizophrenia[6] and alcoholism[5]. The pharmacology of β-carbolines has been reviewed[13–15].

Until now, the analysis of β-carbolines has been performed by means of gas chromatography with mass spectrometric[1,2,5] or flame ionization detection[1], thin-layer chromatography with fluorometric detection[6], mass spectrometry (MS)[3,16] or spectrofluorometry[17]. These methods are not suitable for routine analysis, or lack sensitivity and/or selectivity. The extensive sample pretreatment which is necessary is a common drawback, especially for β-carbolines because these compounds seem to be easily formed during sample pretreatment[18]. High-performance liquid chromatography (HPLC), when used in the reversed-phase mode, together with selective detection, *viz.*, spectrofluorometry or amperometry, has the advantage that only minimum sample pretreatment is necessary.

Recently, the first studies on HPLC analysis of β-carbolines were published[6,19,20]. The separation between a number of β-carbolines was investigated only in one of these studies[19]. For the characterization of four β-carbolines three different HPLC systems were needed. The purpose of our investigation was the development of an HPLC system for the separation of ten β-carbolines (see Table I) together with a selective and sensitive detection system allowing the detection of the β-carbolines in the picogram to nanogram range. The β-carbolines were selected on the basis of their probability of formation *in vivo*.

The chromatographic system described in this paper is adapted from systems developed previously for the bioanalysis of catecholamines, serotonin and their precursors and metabolites in brain tissue and urine samples[21–25]. This reversed-phase ion-pair partition chromatographic (RPIPPC) system uses tri-*n*-butyl phosphate (TBP) as the liquid stationary phase and perchlorate as ion-pairing agent for compounds with an amine function.

EXPERIMENTAL

Apparatus

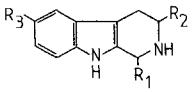
The liquid chromatograph was constructed from custom-made and commercially available parts and consisted of a constant-flow pump (Constametric I; LDC, Riviera Beach, FL, U.S.A.), a thermostatted eluent reservoir, a Bourdon-type manometer, a 7120 injection system (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop and a thermostatted stainless-steel column (10 cm × 3 mm). Detection was performed either by UV spectroscopy (Spectroflow SF 770; Schoeffel Instrument Corporation, Westwood, NJ, U.S.A.) or by fluorometry (SFM 23 LC; Kontron, Zurich, Switzerland). Columns were packed by means of a high-pressure air-amplifier booster pump (DSHF-302; Haskell, Burbank, CA, U.S.A.).

Chemicals and reagents

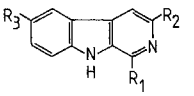
Tryptophan, tryptamine, glyoxylic acid, boron tribromide and 1-Me-BC (see Table I for abbreviations of the β-carbolines) were purchased from Janssen Chimica (Beerse, Belgium). 5-Methoxytryptamine, BC, THBC and 1-Me-THBC-3-COOH were purchased from Sigma (St. Louis, MO, U.S.A.). All chemicals were of analytical

TABLE I

STRUCTURES OF THE TEN β-CARBOLINES



1,2,3,4-Tetrahydro-β-carbolines



β-Carbolines

R_1	R_2	R_3	*Name*
1,2,3,4-Tetrahydro-β-carbolines (THBCs)			
H	H	H	THBC
CH_3	H	H	1-Methyl-THBC (1-Me-THBC)
H	COOH	H	THBC-3-Carboxylic acid (THBC-3-COOH)
CH_3	COOH	H	1-Methyl-THBC-3-carboxylic acid (1-Me-THBC-3-COOH)
H	H	OH	6-Hydroxy-THBC (6-OH-THBC)
CH_3	H	OH	6-Hydroxy-1-methyl-THBC (6-OH-1-Me-THBC)
H	H	OCH_3	6-Methoxy-THBC (6-MeO-THBC)
CH_3	H	OCH_3	6-Methoxy-1-methyl-THBC (6-MeO-1-Me-THBC)
β-Carbolines (BCs)			
H	H	H	BC
CH_3	H	H	1-Methyl-BC (1-Me-BC)

or reagent grade and were used without further purification, except for water which was purified with a Milli-Q water-purification system (Millipore, Bedford, MA, U.S.A.). TBP was obtained from Janssen Chimica. The chromatographic support material, Polygosil C_{18} (mean particle size 5 μm), was obtained from Macherey & Nagel (Düren, F.R.G.).

Syntheses

1-Me-THBC and 6-MeO-1-Me-THBC were synthesized according to a method of Akabori and Saito[26], from tryptamine and 5-methoxytryptamine respectively. 5-Methoxytryptamine was also the starting material for the synthesis of 6-MeO-THBC[27]. 6-OH-THBC and 6-OH-1-Me-THBC were prepared from 6-MeO-THBC[28] and 6-MeO-1-Me-THBC respectively. THBC-3-COOH was synthesized from tryptophan[29].

The purity of all synthesized compounds was determined by reversed-phase (RP) as well as normal phase (NP) chromatography. The RP system was a RPIPP system with TBP loaded on Polygosil C_{18} as the stationary phase and phosphate buffer (pH 2.5)–methanol (8:2) (total phosphate concentration 0.05 *M*; total perchlorate concentration 0.05 *M*) as the mobile phase. The NP system consisted of silica gel Si 100, 8 μm (Merck, Darmstadt, F.R.G.), as the stationary phase and methanol–25% ammonia (100:0.4) and dichloromethane–methanol–25% ammonia (80:20:0.4) as the mobile phases. Detection in both NP and RP chromatography was performed by means of spectrofluorometry and UV spectroscopy.

According to the results, 6-MeO-1-Me-THBC, 6-OH-THBC and 6-OH-1-Me-THBC were impure. The purification of these β-carbolines was carried out by means of a semi-preparative NP system with silica gel Si 60, 8 μm (Merck), as the stationary phase and dichloromethane–methanol–25% ammonia (80:20:0.4) as the

mobile phase (column dimensions: 25 cm × 9.5 mm; flow-rate: 3.9 ml/min). The identity of all synthesized and purified compounds was confirmed by means of ^{1}H NMR and mass spectrometry.

Chromatography

The column packing procedure was based on a slurry technique as described elsewhere[30]. 1,1,1-Trichloroethane and methanol were used as dispersing and displacing solvent respectively. After the column had been washed with about 100 ml of water, the mobile phase (saturated with TBP) was pumped through and the column was loaded *in situ* by injection of 20-μl aliquots of TBP until supersaturation of the column effluent was observed. The porosity of the loaded column was about 0.55.

Due to a possible supersaturation of the mobile phase with TBP, small droplets of TBP can occur, which hampers the detection. This supersaturation can be avoided if the mobile phase is prepared as follows:

(1) Remove the air from the solvent

(2) Adjust the temperature

(3) Add TBP to the solvent by syringing 1-ml portions (2–5 portions per litre). Small TBP droplets must be present throughout the solvent, which can be achieved by stirring gently for a few minutes (In order to prevent emulsification, vigorous stirring should be avoided.)

(4) Leave the solvent without stirring. After 15–30 min the mobile phase is ready for use. Stir gently during use.

The capacity ratio, k'_i, of a compound i was determined from the retention time, t_{Ri}, and the retention time of an unretained compound, t_{R0}, for which sodium iodide (injection of 20 μl of a 1 mM solution; detection by UV spectroscopy) was used.

The optimum wavelengths for the detection of the various compounds were determined by stopped-flow scanning (both for fluorometric and UV spectroscopic detection).

THEORETICAL

The mechanism of retention of charged and neutral compounds in RPIPP systems based on TBP as the stationary phase has been discussed previously[21,22,25,31].

In the studied pH range the compounds of interest can have one or two charged groups. In the first class of compounds, the amines (*viz.*, all the compounds except THBC-3-COOH and 1-Me-THBC-3-COOH), only the nitrogen atom numbered 2 (see Fig. 1) can be protonated. In the second class of compounds, the "amino acids" (*viz.*, THBC-3-COOH and 1-Me-THBC-3-COOH), the carboxyl group on the carbon atom numbered 3 can also dissociate.

The amines can be present in their protonated (BH^+) or deprotonated (B) form. The protonated amines can form ion pairs with perchlorate ions (X^-):

$$BH^+_{aq} + X^-_{aq} \overset{K_{ip}}{\rightleftarrows} BHX_{aq} \qquad (1)$$

Ion-pair extraction into the organic phase involves adduct formation with TBP (S)[22]:

$$BHX_{aq} + nS_{org} \overset{K_{ex,1}}{\rightleftharpoons} (BHXS_n)_{org} \quad (2)$$

The deprotonated amines are retained as such; extraction can involve adduct formation:

$$B_{aq} + nS_{org} \overset{K_{ex,2}}{\rightleftharpoons} (BS_n)_{org} \quad (3)$$

From the equilibria 1–3 an expression can be derived for the distribution coefficient, D_{amine}:

$$D_{amine} = \frac{[BHXS_n]_{org} + [BS_n]_{org}}{[BH^+]_{aq} + [BHX]_{aq} + [B]_{aq}} = \frac{K_{ex,1}K_{ip}[S]^n_{org}[X^-]_{aq} + K_{ex,2}[S]^n_{org}K_a([H^+]_{aq})^{-1}}{1 + K_{ip}[X^-]_{aq} + K_a([H^+]_{aq})^{-1}} \quad (4)$$

where K_a is the conditional acid dissociation constant for the amine function in the molecule.

Amino acids can theoretically be present in four different forms. In the pH range studied (see Results and Discussion) the amine function is always protonated and thus only two forms have to be taken into account, *viz.*, $^+$HBAH and $^+$HBA$^-$. For both forms ion-pair formation with perchlorate ions will take place, but only the XHBAH ion pair will contribute to the retention. For the distribution coefficient the following expression can be derived (see eqn. 5 in ref. 25):

$$D_{amino\ acid} = \frac{[XHBAHS_n]_{org}}{[XHBAH]_{aq} + [^+HBAH]_{aq} + [XHBA^-]_{aq} + [^+HBA^-]_{aq}} = \frac{K_{ex}[S]^n_{org}K_{ip,1}[X^-]_{aq}}{K_{ip,1}[X^-]_{aq} + 1 + K_a([H^+]_{aq})^{-1}(1 + K_{ip,2}[X^-]_{aq})} \quad (5)$$

In this equation $K_{ip,1}$ and $K_{ip,2}$ are the ion-pair formation constants of $^+$HBAH and $^+$HBA$^-$ respectively, K_{ex} is the extraction constant of the XHBAH ion pair and K_a is the conditional acid dissociation constant.

RESULTS AND DISCUSSION

Chromatography

The retention of amines and amino acids in RPIPP systems can be influenced by different parameters, such as the pH, the counter-ion concentration and the modifier content of the mobile phase and the temperature of the phase system[25]. The influence of the three mobile phase parameters was systematically investigated.

The influence of the pH of the mobile phase on retention is depicted in Fig.

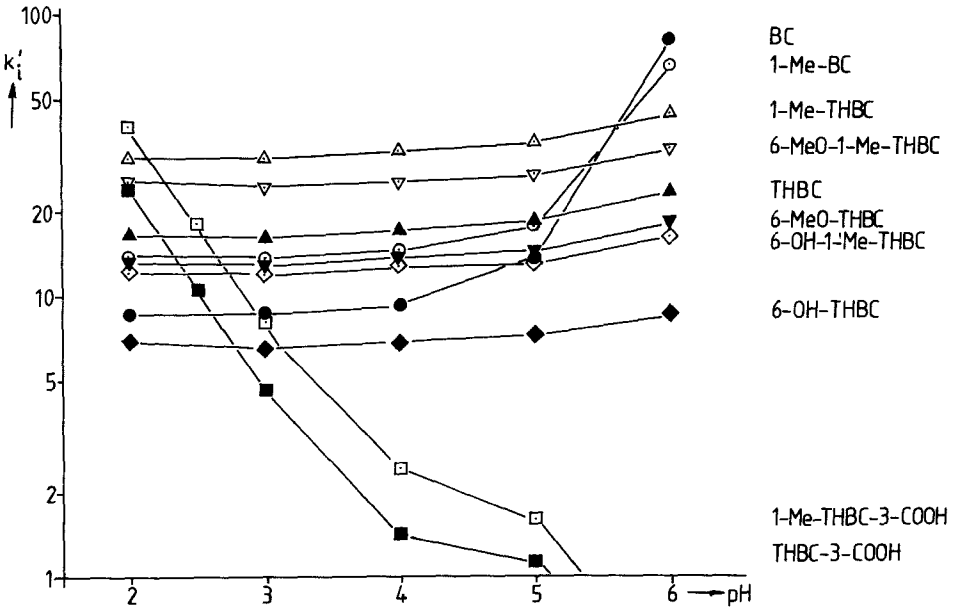


Fig. 2. Dependence of the capacity ratios on the pH of the aqueous part of the mobile phase. Mobile phase: phosphate buffer–methanol (8:2); total concentration of perchlorate and buffer, 0.05 *M*. Temperature 303°K.

2. As expected, the only compounds whose retention was influenced to an appreciable extent were those with a pK_a value in or near the pH range investigated.

According to eqn. 5 for amino acids, the curve of log k' *vs.* the pH of the mobile phase should contain two linear parts, *viz.*,

$$\log D = \log (\text{numerator eqn. 5}) - \log (K_{ip,1}[X^-]_{aq} + 1) \quad (6)$$

if $[H^+]_{aq} \gg K_a$ (horizontal line) and

$$\log D = \log (\text{numerator eqn. 5}) - \log K_a - \text{pH} - \log (K_{ip,2}[X^-]_{aq} + 1) \quad (7)$$

if $[H^+]_{aq} \ll K_a$ (line with a negative slope). In Fig. 2 only the second part of the curve can be seen indicating that the value of pK_a must be smaller than about 2. As far as we know, the pK_a values of THBC-3-COOH and 1-Me-THBC-3-COOH have not been reported, but should be about the same as that of proline which is 1.95 (ref. 32, p. J 115). According to eqn. 7 the slope of the log k' *vs.* pH curve should have a value of -1. Even in the steepest range (pH 2–3, see Fig. 2) the slope does not exceed a value of about -0.7. A similar behaviour has been observed previously for acids and amino acids derived from tryptophan in a mobile phase containing 20% methanol[25]. At higher pH values (>3) for the amino acids, the slope of the log k' *vs.* pH curve decreases, while between pH 5 and 6 the slope increases again (see Fig. 2).

As regards their pH behaviour, the amines can be divided into two groups,

viz., the BCs and the THBCs. The pK_a values of the BCs and THBCs are comparable to those of pyridine (pK_a = 5.2; see ref. 32, p. J 120) and piperidine (pK_a = 11.1; see ref. 32, p. J 114) respectively. According to theory (see eqn. 4), the retention of the amines will only change if the pH of the mobile phase approaches the pK_a. Indeed, the retention of the THBCs is hardly affected by the pH, and the retention of the BCs increases at pH values of about 5 (see Fig. 2). This indicates that the uncharged amines (B) are more strongly retained than the ion pairs of the protonated amines (BHX). The reversal of the order of elution of the two BCs at high pH values should also be ascribed to a difference in their extraction constants.

Fig. 3 shows, in a semi-logarithmic plot, the influence of the percentage of methanol in the mobile phase on the retention. Obviously, for the β-carbolines the use of a modifier is necessary because otherwise the retention would be too high. However, only minor changes in selectivity can be obtained by variation of this mobile phase parameter. The behaviour of the two amino acids is not shown in this plot because for these compounds no relevant information is obtained: addition of methanol to the mobile phase (which is initially buffered to pH 2.0) will influence the pH, to an extent dependent on the amount of methanol added. At a pH of about 2 a small change in the pH of the mobile phase does not affect the retention of the amines but results in a profound effect on the retention of the amino acids. It should be emphasized that methanol contents above 30% should be avoided because of the risk of unstable phase systems and consequently noisy detector baselines.

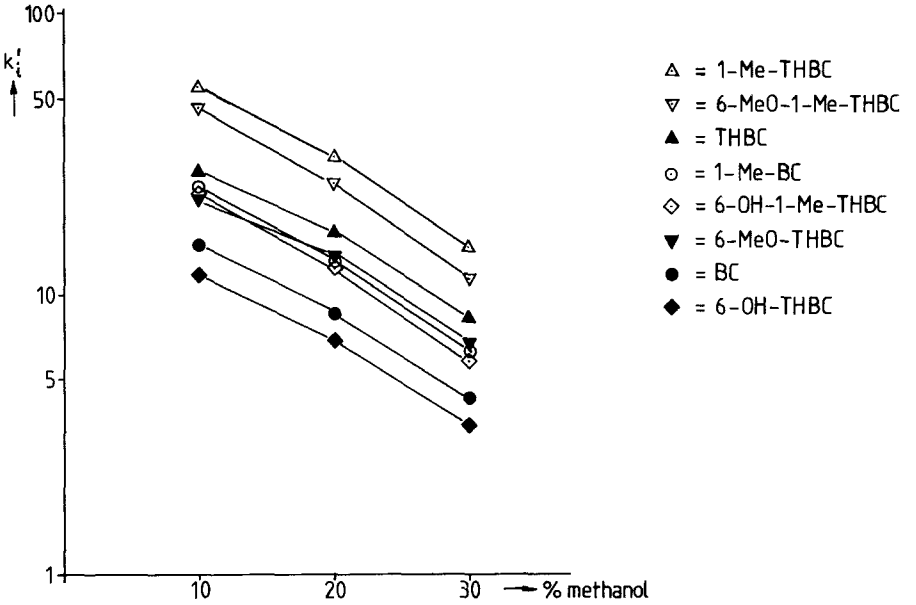


Fig. 3. Influence of the methanol content of the mobile phase on the capacity ratios. Mobile phase: phosphate buffer (pH 2.0) with various percentages of methanol; total perchlorate and buffer concentrations, 0.05 *M*. Temperature 303°K.

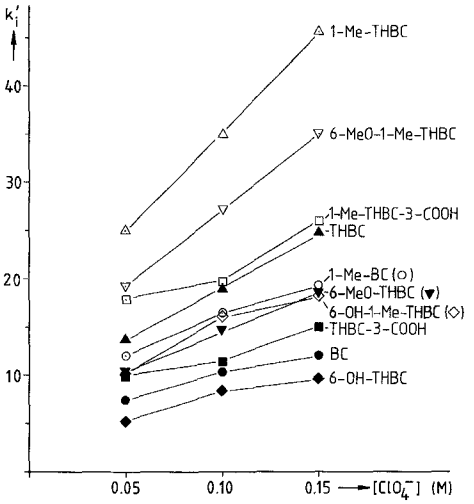


Fig. 4. Effect of the total counter-ion concentration of the mobile phase on the capacity ratios. Mobile phase: phosphate buffer (pH 2.5)–methanol (8:2); total buffer concentration, 0.05 *M*. Temperature 303°K.

Fig. 4 shows the relationship between counter-ion concentration and retention. As expected the retention of all the β-carbolines increases with the perchlorate concentration. For four compounds, *viz.*, the BCs and the 6-OH-THBCs, the shape of the curve is slightly convex; for the other amines the retention increases linearly with the counter-ion concentration. According to theory both shapes can be explained. At pH 2.5 the amines are completely protonated and thus eqn. 4 can be simplified to:

$$D_{\text{amine}} = \frac{K_{\text{ex},1} K_{\text{ip}} [\text{S}]^n_{\text{org}} [\text{X}^-]_{\text{aq}}}{1 + K_{\text{ip}} [\text{X}^-]_{\text{aq}}} \tag{8}$$

Depending on the value of $K_{\text{ip}}[\text{X}^-]_{\text{aq}}$, the relationship between D_{amine} (and thus the capacity ratio, k') and the perchlorate concentration in the mobile phase is linear (if $K_{\text{ip}}[\text{X}^-]_{\text{aq}} \ll 1$) or convex (if $K_{\text{ip}}[\text{X}^-]_{\text{aq}} \approx 1$).

In previous studies straight lines were obtained for catecholamines in mobile phases without modifier[21–23] and convex curves were found for β-indole-3-ethylamines in a mobile phase containing 20% (v/v) methanol[25]. Although no definitive conclusions can be drawn, because in this study only three counter-ion concentrations were employed, the results suggest that for β-carbolines even in mobile phases containing 20% methanol, the K_{ip} values are rather small (and thus $K_{\text{ip}}[\text{X}^-]_{\text{aq}} \leqslant 1$, see above), indicating that ion pairs are not easily formed in the mobile phase.

For the two amino acids a concave relationship between k' and the counter-ion concentration is observed (see Fig. 4). No conclusions can be drawn from these plots because the pH of the mobile phase approaches the pK_a of the amino acids (see above) and thus the counter-ion effect is easily masked by a pH effect.

From the results depicted in Figs. 2–4 the optimum conditions for the separation of the ten β-carbolines can be derived. The retention is only selectively influenced by a change of the pH of the mobile phase. From Fig. 2 it is seen that at a pH

of 3.5 most β-carbolines are separated from each other while all the components are still sufficiently retained. Fig. 5 shows in two chromatograms the retention of the ten β-carbolines in the optimized system. 6-OH-1-Me-THBC and 6-MeO-THBC cannot be separated in the RPIPP system. Furthermore there is no baseline separation between these two compounds, 1-Me-BC and THBC. The chromatograms in Fig. 5 were obtained with a mobile phase containing 0.05 *M* diethylamine. This basic solvent was added in order to suppress peak tailing, which is probably caused by interaction of the β-carbolines with underivatized silanol groups of the support material. Diethylamine gives rise to a small decrease in retention but does not influence the selectivity of the system. As is seen in Fig. 5, analysis times are about 13 min.

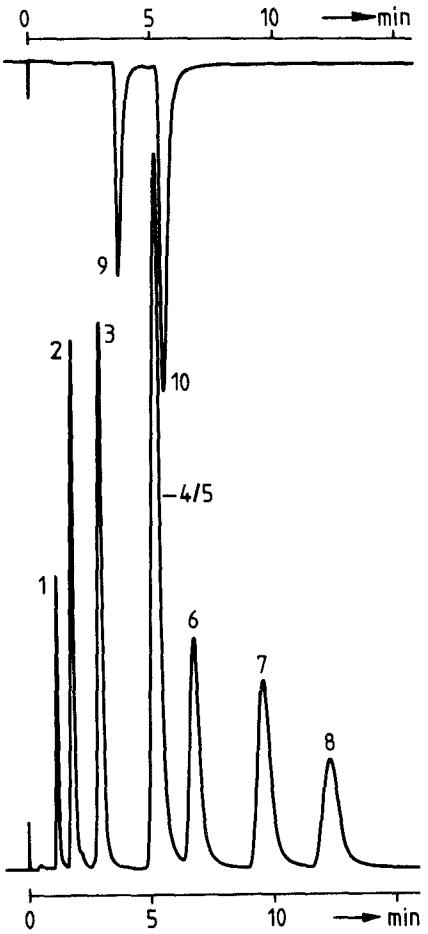


Fig. 5. Separation of the ten β-carbolines under optimized conditions. Mobile phase: phosphate buffer (pH 3.5)–methanol (8:2); total perchlorate, buffer and diethylamine concentration, 0.05 *M*. Temperature 308°K. Flow-rate 1.0 ml/min. Detection: fluorometric, lower trace: excitation wavelength = 290 nm, emission wavelength = 350 nm; upper trace: excitation wavelength = 302 nm, emission wavelength = 420 nm. Compounds: 1 = THBC-3-COOH; 2 = 1-Me-THBC-3-COOH; 3 = 6-OH-THBC; 4 = 6-OH-1-Me-THBC; 5 = 6-MeO-THBC; 6 = THBC; 7 = 6-MeO-1-Me-THBC; 8 = 1-Me-THBC; 9 = BC; 10 = 1-Me-BC.

Detection

Detection can be performed by means of UV spectroscopy or fluorometry. UV detection is rather insensitive with detection limits in the nanogram range. Fluorometric detection offers more favourable detection limits and has the additional advantage of being far more selective. This is clearly shown in Fig. 5. The same mixture of ten β-carbolines has been injected twice. By appropriate choice of the excitation and emission wavelengths only the BCs or the THBCs are detected. Thus, the determination of 1-Me-BC and THBC can be performed without any problem despite the fact that these compounds are not completely separated from each other and from the preceding peak. The optimum wavelengths for fluorometric detection are summarized in Table II. The maximum excitation and emission wavelengths are shifted by about 10 and 20 nm respectively when the molecule is substituted with a hydroxy or methoxy group at the carbon atom numbered 6. The same kind of behaviour was found previously for indoles[25]. Unfortunately, at the optimum wavelengths for detection of the 6-OH- and 6-MeO-substituted compounds the decrease in fluorescence for the unsubstituted compounds is 30–40% and *vice versa*. If it is desirable to detect compounds of both groups simultaneously, excitation and emission wavelengths of 280 and 330 nm respectively are the best compromise. At these wavelengths the loss of signal for the 6-substituted and 6-unsubstituted compounds is only 5 and 20% respectively.

TABLE II

OPTIMUM WAVELENGTHS FOR FLUOROMETRIC DETECTION

For conditions, see Fig. 5.

Compound	λ_{ex} *(nm)*	λ_{em} *(nm)*
THBC-3-COOH, 1-Me-THBC-3-COOH, THBC, 1-Me-THBC	280	350
6-OH-THBC, 6-MeO-THBC, 6-OH-1-Me-THBC, 6-MeO-1-Me-THBC	290	330
BC	300	440
1-Me-BC	300	425

Based on a signal to noise ratio of 3, the detection limits range from 15 pg for the first compound eluted (THBC-3-COOH) to 150 pg for the last (1-Me-THBC). The linear dynamic range, which was determined for THBC and 6-OH-THBC, is about four decades.

CONCLUSIONS

RPIPPC with TBP as stationary phase and perchlorate as counter ion yields rapid separation of β-carbolines. The retention can be influenced selectively by means of the pH of the mobile phase, while the perchlorate concentration and methanol content of the mobile phase and the temperature of the phase system affect the retention of all the compounds in the same way. In general, the relationship between these parameters and the retention of the β-carbolines is in agreement with theory.

Using fluorometric detection, eight β-carbolines can be analysed without any problem with maximum analysis times of about 13 min. Fluorometric detection,

which is more sensitive and selective than UV detection, offers detection limits ranging from 15 to 150 pg. Future research will be devoted to the application of this chromatographic system to the assay of β-carbolines in biological material, *i.e.*, blood platelets.

ACKNOWLEDGEMENTS

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