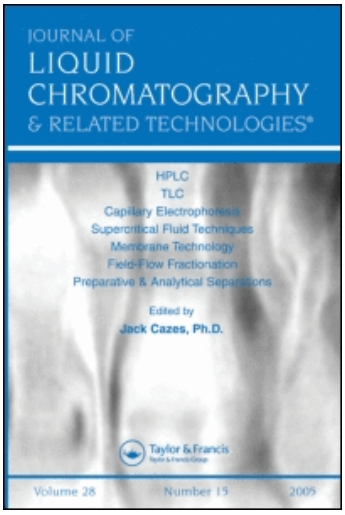

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QUANTITATIVE DETERMINATION OF TABERSONINE AND METHOXYTABERSONINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for the quantitative determination of tabersonine and methoxytabersonine by reversed-phase high-performance li - quid chromatography has been developed based on employing amine modifiers of the mobile phase as silanol group masking agents. Thus peak tailing is reduced and peak shapes are improved. The effect of pH of the eluent and the type of the organic modifier upon the overall separation has been examined. A mobile phase optimization procedure has been carried out for selecting the most favorable eluent mixture. The accuracy and precision achieved by the quantitative analysis are satisfactory.

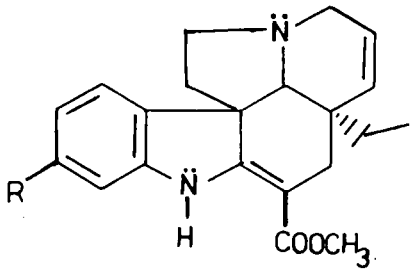
INTRODUCTION

Tabersonine(T) and methoxytabersonine(MT), two indole alkaloids of the Aspidosperma type are potential presursors of

the alkaloid vindoline during its biosynthesis (1). The significance of the latter alkaloid is determined by its presnce as an indoline half in the dimeric molecule of the antitumor alkaloid vinblastine.

The conversion of T into vindoline (2) has been recently reported as well as the synthesis of 17-substituted MTs in order to obtain modified vindolines (3). The latter would eventually serve as an initial substance for the synthesis of vinblastine-like compounds.

MT has been found for the first time in the plant Vinca herbacea (4). It composes together with T and lochnerinine the nonpolar part of the total alkaloid amount. The content of MT in the plant during its growth stages proved to be of interest. In this regard we developed an analytical method for the quantitive determination of T and MT by reversed-phase high-performance liquid chromatography (RP-HPLC).



T : R=H
MT: R=OCH3

T and MT are basic compounds with similar physico-chemical properties. Thus it was necessary to be determined the effect upon resolution (R) of the type and concentration of the amine modifiers in the mobile phase along with the structure of the stationary phase. Resolution in general can be affected by three independent factors: selectivity - α , column efficiency - N and capacity factor (solvent strength) - k. Owing to the hydrophobic character of T and MT C_8 or C_{18} reversed-phase columns would prove suitable for their separation. These stationary phases, however, posses a great deal of unsilanized silanol groups - up to 50 % (5) of the total number of silanols and demonstrate a pronounced adsorption towards basic compounds. They manifest such an adsorption even after endcapping (6) because unreacted silanols are still left. This undesired adsorption can be surmounted by modifying the liquid phase with amines which mask the silanol groups (7,8,9,10). It was shown in a recent investigation that the type and the concentration of the amine modifiers and the organic solvents in the liquid phase influence to a great extent not only the adsorption of the silanol groups but the mass transfer of the column also (11). It was determined that the values of the resolution achieved between T and MT are higher when two amine modifiers possessing complementary properties are employed in comparison with those achieved when the same amines are used individually.

Considering the mobile phase optimization the overlapping resolution mapping (ORM) scheme (12,13,14) proved to be the most rational approach for the purpose. In relation to other methods the ORM enables the optimization to be carried out by a relatively small number of experiments.

Owing to the fact that T and MT are bases it was necessary the relationship between the capacity factor k and pH of the mobile phase to be determined. The relationship $k=f(pH)$ would point out the range of pH values in which k would not be dependent upon pH. This would increase the accuracy and the precision of the chromatographic analysis.

EXPERIMENTAL

All chromatographic analyses were performed on an Isco model 2350 and a V 4 Variable Absorbance Detector (Isco,Lincoln, Nebraska,U.S.A.) set at 228 nm. Samples were injected with a Valco C6W (Valco Instruments Co. Inc.,Houston,Texas,U.S.A.) with a 25 µl loop. Chromatographic data was collected and analyzed on an IBM - PC - AT with Chemresearch Data Management Software. The dead volume of the chromatographic system was determined by methanol as the unretained component for all mobile phases. The UV-spectra of T, MT and the internal standard colchicine were obtained on a Perkin-Elmer LC-253 Diode Array Detector (Norwalk, Connecticut,U.S.A.) and showed that the optimum wavelength is $\lambda = 228$ nm.

Two reversed-phase columns were employed for the analyses: a home-packed (250 x 4.5 mm I.D.) with 5 μm Nucleosil C_8 (Macherey-Nagel,Duren,F.R.G.) and a Perkin-Elmer (150 x 4.5 mm I.D.) C_8, 5 μm (Norwalk,Connecticut,U.S.A.). All chromatography was carried out at ambient temperature (22 C).

The organic solvents employed as mobile phase components - methanol, acetonitrile and tetrahydrofuran (Merck,Darmstadt, F.R.G.) were of HPLC-grade. Water was bidistilled and filtered through a 0.5 μm Millipore membrane filter. All mobile phases were degassed in an ultrasonic bath prior to use. Diethylamine (DEA) and dibuthylamine (DBA) of purities better than 99 % were obtained from Merck (Darmstadt,F.R.G.). After mixing the organic solvents with water and adding the amine modifiers pH was adjusted by phosphoric acid and controlled with a Praci-tronic MV870 Digital pH-meter (G.D.R.).

Samples of the analyzed alkaloids were prepared by dissolving in analytical-grade ethanol obtained from Merck (Darmstadt, F.R.G.). Owing to the possibility of light and temperature induced degradation of MT the solutions were stored in a refrigerator.

T and MT were isolated from Vinca herbacea and purified by column liquid chromatography as described in (4). Their struc - tures were confirmed by mass spectrometry, thin-layer chromatography, ultraviolet and infrared spectroscopy and nuclear magnetic resonance.

The data necessary for plotting the calibration graphs was obtained by the following chromatographic conditions: home - packed column (250 x 4.5 mm I.D.) with Nucleosil C_8, 5 μm ; mobile phase: methanol-acetonitrile-water (25: 5:70, v/v/v), 0.5 % DBA, 0.2 % DEA; pH = 3.2 ± 0.2; flowrate 1 ml/min; UV de - tection at 228 nm.

RESULTS AND DISCUSSION

A preliminary determination of the efficiency of the used reversed-phase columns was carried out. Over 10 000 theoretical plates/m and k = 6 were obtained for acetophenone eluted with methanol-water (60:40, v/v). Regarding the capacity factors k for T and MT it was necessary their values to be maintained within the range 1-10 (15). Another requirement considering k was that it should not be dependent on pH of the mobile phase. This was the reason why we determined the relationship k = f(pH) for the mobile phase employed for generating the calibration graphs by changing its acidity with phosphoric acid (Fig. 1). It is evident that within the pH range of 3-4 k does not display a dependence upon the acidity, thus all chromatographic experi - ments were performed at a pH of the mobile phase 3.2 ± 0.1 .

In a preceding study we showed that the separation of T and MT achieved with DEA and DBA as mobile phase additives is better than the one for the case when the same amines are em-

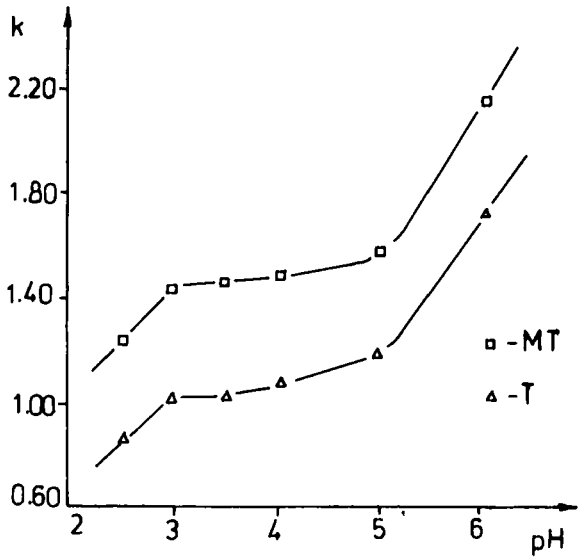


FIGURE 1. Dependence of the capacity factor k upon changes in pH of the mobile phase.

ployed separately. The reason is that DEA more easily penetrates and overlaps the silanol groups while DBA adheres to the hydrocarbon ligands, thus forming a complex which changes their hydrophobicity and improves the transfer between the mobile phase and the support. As a prove serves the separation of T and MT from the more polar alkaloids (unidentified) present in the real samples (Fig. 2). In the case of using as a mobile phase modifier a mixture of 0.1 % DBA and 0.2 % DEA the separation of T and MT is R=1.70 but the peak of T overlaps partially with the polar alkaloids (Fig. 2a). The increasing of DBA 's concentration to 0.5 % leads to achieving of a bet-

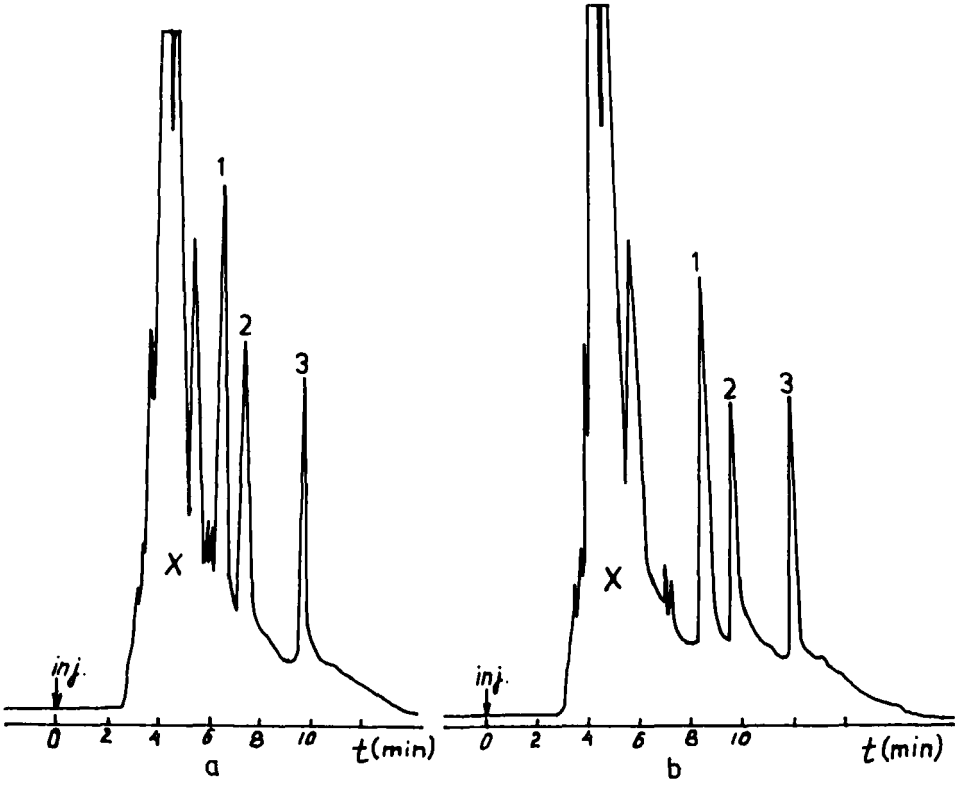
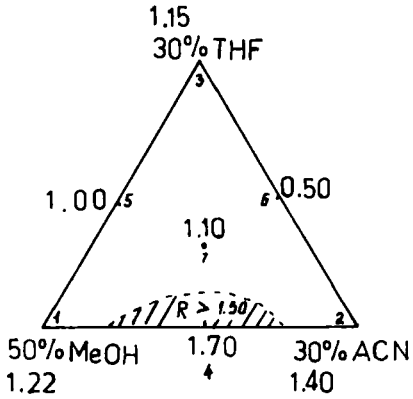


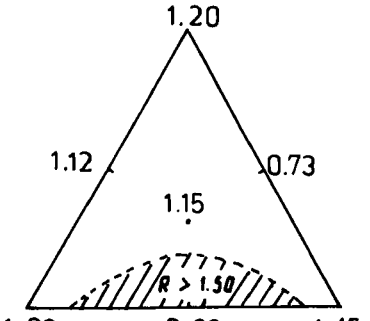
FIGURE 2. Chromatograms of unidentified alkaloids (X), tabersonine (1), methoxytabersonine (2) and colchicine (3); Nucleosil C_8 (250 x 4 mm I. D.) 5 µm, acetonitrile - methanol - water (25: 5:75, v/v/v), 1 ml/min, λ= 228 nm , pH=3.2; a - 0.2 % diethylamine, 0.1 % dibuthylamine ; b - 0.2 % diethylamine, 0.5 % dibuthylamine.

ter separation between T and MT on one hand and between T and the rest of the alkaloids on the other (Fig. 2b). This occurs in spite of the fact that due to the higher concentration of DBA the mobile phase strength is greater, hence T and MT should be eluted faster.

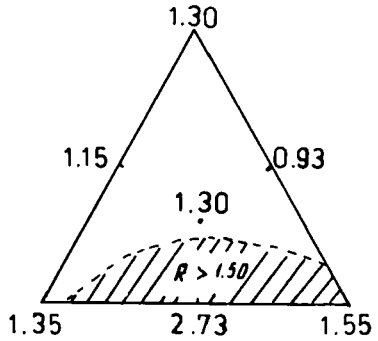
A mobile phase optimization procedure was carried out by the ORM scheme. As a criterion for the optimum composition



0.2% DEA
0.1% DBA



0.2% DEA
0.3 % DBA



0.2% DEA
0.5% DBA

FIGURE 3. Chromatographic resolution maps for tabersonine and methoxytabersonine representing the effect upon resolution of the concentration of dibuthylamine and the composition of the mobile phase.

of the eluent served the value of the resolution between the peaks of T and MT. By mixing different concentrations of the three organic solvents in water: methanol (50 %), acetonitrile (30 %) and tetrahydrofuran (30 %) and taking the Snyder selectivity triangle into consideration we determined that by increasing DBA 's concentration in the mobile phase the separation of T and MT improved (Fig. 3). The best resolution achieved with a mobile phase consisting of only one organic solvent (in water) was R=1.55 for the case of acetonitrile - water (30:70,v/v). The surface of the triangle corresponding to a resolution R $\geq$1.50 expands upon increasing the concentration of DBA, i. e. it is possible to achieve higher resolution by a greater variation in the type and concentration of the solvents composing the mobile phase.

Data on the accuracy and precision of the liquid chromatographic quantitative determination of T and MT in model mixtures with colchicine as an internal standard are presented in Table I. The concentrations of T and MT in the model mixtures were chosen to be near those usually found in the investigated samples. Calibration graphs were generated by plotting the peak height ratio verses the concentration of the solutes in the range of 0.2 - 2.0 mg/ml. In all instances the calibration graphs were linear with a correlation coefficient of .995 passed through the origin. Values of unknown sample concentrations were determined by comparison with the calibration graphs.

TABLE I

Precision and Accuracy of the Liquid Chromatographic Determination of Tabersonine and Methoxytabersonine

Concentration(mg/ml) Model mixture		Calculated value ± standard deviation		Precision, V (%) [a]		Accuracy, A (%) [b]	
T	MT	T	MT	T	MT	T	MT
0.24	0.23	0.28 ± .02	0.24 ± .01	5.94	7.08	16.67	4.35
0.73	0.85	0.78 ± .03	0.93 ± .03	3.28	3.53	6.85	9.41
1.02	1.17	1.09 ± .02	1.12 ± .03	1.81	2.75	6.86	4.27
1.90	1.16	2.08 ± .03	1.91 ± .02	1.27	1.33	9.47	8.52

[a] $V\ (\%) = (S/\bar{x}) \cdot 100$ $(n = 7)$; S - standard deviation; $\bar{x}$ - mean of the calculated values for a given concentration

[b] $A\ (\%) = \left| \frac{\text{actual concentration - calculated concentration}}{\text{actual concentration}} \right| \cdot 100$

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