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QUANTITATIVE ANALYSIS OF *d*-TUBOCURARINE CHLORIDE IN CURARE BY COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

An improved quantitative analysis of *d*-tubocurarine chloride in the plant extract curare is presented. Gradient high-performance liquid chromatography on a hydrophobic stationary phase was found to be very suitable for the analysis of quaternary ammonium bases such as the complex mixture of curare alkaloids. Owing to the residual free silanol groups on the modified silica surface, the curare alkaloids are eluted from a reversed-phase column only if an electrolyte is added to the mobile phase.

In order to optimize the separation, the effects of pH, the nature of the cation in the buffer and the concentration of the buffer on the retention of the alkaloids were investigated. Using a tetramethylammonium phosphate buffer at pH 4 in a gradient of water–methanol, undesirable retardation effects on the reversed-phase column could be suppressed sufficiently. As a result, an accurate method for the determination of *d*-tubocurarine chloride in curare was obtained. The coefficient of variation of this analysis is only 1.3%.

INTRODUCTION

Chondrodendron tomentosum (Ruiz and Pavon) is one of the menispermaceous plants used by South-American Indians for preparing the arrow poison curare[1,2]. Curare is the evaporation residue of the plant extract obtained by cooking the plant pulp with water. It is used as a commercial source of the alkaloid *d*-tubocurarine chloride (I)[3,4].

d-Tubocurarine chloride (I), which exerts a muscle-relaxing effect, is useful in surgical operations and during shock therapy for certain mental diseases. However, because of the narrow safety margin with *d*-tubocurarine chloride, it is essential that

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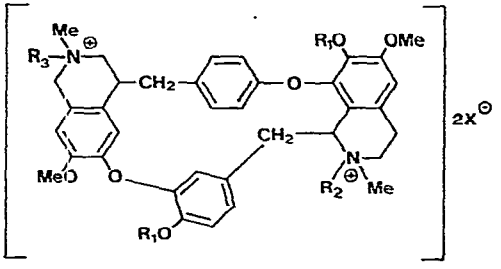
it should be used in a substantially pure form in order to eliminate the danger of an overdose owing to incorrect standardization of the product.

A quantitative analytical method for *d*-tubocurarine chloride is therefore of great importance. Moreover, it is of interest to gain a better insight into the composition of curare itself, and particularly the amount of *d*-tubocurarine chloride present in it. Previously polarimetry and thin-layer chromatography[5–7] were applied for the quantitative determination of *d*-tubocurarine chloride in curare. The former method is non-specific and, as curare contains several other, closely related, alkaloids that also show optical rotation, this method is rather speculative. Thin-layer chromatographic methods for the separation of curare alkaloids give results with a poor reproducibility; moreover, the quantification is rather cumbersome.

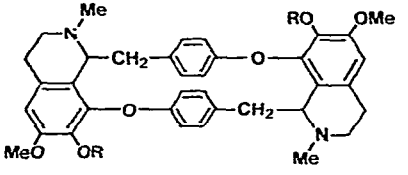
The low working temperature and the large number of possibilities for adjusting the selectivity of the phase system combined with high efficiencies make high-performance liquid chromatography (HPLC) very suitable for the analysis of the thermally labile and complex curare alkaloids.

Several column liquid chromatographic separations of alkaloids have been described[8–18], but all were related to tertiary alkaloid bases. The absence of methods for the liquid chromatographic separation of quaternary alkaloids is caused by the very high polarity of these compounds and the limited number of suitable solvents.

In this study, the five most important curare alkaloids were available as standards. The structures of these alkaloids are given below.



1: HX $R_1=R_3=HR_2=Me$ (+-) tubocurarine chloride
2: 2HX $R_1=R_2=R_3=H$ (+-) chondrocurine
3: $R_1=HR_2=R_3=Me$ (--) curarine
5: 2HX $R_1=R_2=R_3=H$ (--) curine



4: R=H (++) isochondrodendrine

The quaternary curare alkaloids are soluble only in water and very polar organic solvents such as methanol, ethanol and acetone and in mixtures of them. On the other hand, there is a great difference in the polarities of the various curare

alkaloids (see structures). From these data, it is appropriate to choose a phase system with a hydrophobic stationary phase and a gradient of water–methanol as the mobile phase.

In this paper, the development of a quantitative HPLC method for the determination of *d*-tubocurarine chloride (1) in curare is described.

EXPERIMENTAL

Apparatus

Use was made of a high-performance liquid chromatograph in the gradient mode (Waters, two Model 6000 pumps and a Model 660 solvent programmer), equipped with a UV detector (Waters Model 440), a high-pressure sampling valve (Chromatronix HPSV-20), a linear potentiometric recorder (Kipp & Zonen BD 9, two channel) and a computing integrator system (Spectra Physics SP 4000).

Throughout the investigation a 30-cm μBondapak C_{18} column (Waters Assoc., Milford, Mass., U.S.A.; particle size 10 μm) and various gradients of water–methanol were used. In order to avoid contamination of the column with plant materials, a short pre-column (5 cm × 4.6 mm I.D.) tap-filled with Corasil/C_{18} (Waters Assoc., particle size 37–50 μm) was placed between the injector and the separation column.

Chemicals and materials

In all experiments distilled water and organic solvents of analytical-reagent grade (Baker, Deventer, The Netherlands) were used. Tetramethylammonium hydroxide (TMAH) was used as a 20% solution in methanol (Aldrich, Milwaukee, Wisc., U.S.A.).

The standard alkaloids (2–5) were isolated from curare and their structures established by specific optical rotation measurements, infrared and nuclear magnetic resonance spectroscopy and mass spectrometry.

Procedures

The gradient elution procedure was standardized: after running the appropriate gradient the column was regenerated by the reverse gradient to the starting conditions in 5 min, followed by a re-equilibration of the column under these conditions for 10 min. The column temperature was maintained at 20°. The flow-rate of 1 ml/min was obtained with pressures of 1500–2000 p.s.i.

All samples were dissolved in water to which a few drops of 4 *M* hydrochloric acid had been added. Prior to injection (20 μl) these solutions were subjected to Millipore filtration (sample clarification kit, Waters Assoc.).

The UV spectrum of *d*-tubocurarine chloride in acidic medium has a maximum at about 280 nm, so detection was made at this wavelength.

In the various experiments, relative net retention data [$t_r = (t_a - t_0)/t_0$] were calculated from the retention times of the alkaloids (t_a) and of an unretarded compound (t_0), for which methanol (RI detection) was used.

The following elution systems were used.

(i) Study of the influence of the pH of the mobile phase. A linear gradient from solvent A to B in 45 min. Solvent A was a 0.025 *M* solution of TMAH in water–methanol (90:10) adjusted to pH 3–7 with concentrated orthophosphoric acid; solvent B consisted of the same buffer with the same pH in water–methanol (10:90). For the

study of the influence of the buffer concentration the same elution system was used, but with variable TMAH concentrations.

(ii) Study of the effect of the nature of the cation. A linear gradient of 90% of solvent A and 10% of solvent B to 15% of A and 85% of B in 30 min. Solvent A was a 0.025 *M* solution of potassium, ammonium or tetramethylammonium phosphate buffer in water–methanol (75:25) adjusted at pH 4 with concentrated orthophosphoric acid; solvent B consisted of the same buffer with the same pH in water–methanol (55:45).

(iii) Quantitative analysis. A linear gradient of 90% of solvent A and 10% of solvent B to 15% of A and 85% of B in 30 min. Solvent A was a 0.025 *M* solution of TMAH in water–methanol (75:25) adjusted at pH 4 with concentrated orthophosphoric acid; solvent B consisted of the same buffer with the same pH in water–methanol (55:45).

RESULTS AND DISCUSSION

Preliminary experiments revealed that the curare alkaloids are eluted from a reversed-phase column using water–methanol mixtures only if an electrolyte is added to this mobile phase.

From the literature[19,20], it is known that RP-modified silica still contains some acidic silanol groups. For the strong alkaloid bases, in our opinion the RP-modified silica behaves as a weak ion-exchange resin owing to the silanol groups that are still present. Owing to this effect, the alkaloids are irreversibly bonded on the column in water–methanol.

As would be expected, the addition of even a small amount of acetic acid (1%) to the mobile phase caused the elution of the alkaloids. The alkaloids, however, were eluted in very asymmetric bands from the column, which indicates an insufficient deactivation of the active polar sites on the RP-modified silica by acetic acid. Upon addition of a buffer of appropriate strength the peak performance was substantially improved.

From other work[21] it is known that the extent of the deactivation of the silica is mainly affected by the nature of the cation in the buffer.

In order to find the optimal conditions for the separation and quantitative determination of the curare alkaloids, a systematic investigation was made of the effects on this separation of the pH, the nature of the cation in the buffer and the buffer concentration.

pH of the mobile phase[22,23]

The pH of the mobile phase has a pronounced effect upon the separation of the curare alkaloids, because both quaternary and tertiary bases are present. Under basic conditions the alkaloids and the stationary silica phase are unstable. Hence the influence of the pH on the reversed-phase separation of the alkaloids was investigated in the pH range 3–7. The results are shown in Fig. 1.

The effect of the pH of the eluent on the relative net retention of the five standard alkaloids is two-fold: (i) owing to the protonation of all tertiary amino groups in the alkaloids, the relative net retention decreases with decreasing pH value; and (ii) the sequence of elution changes with the pH.

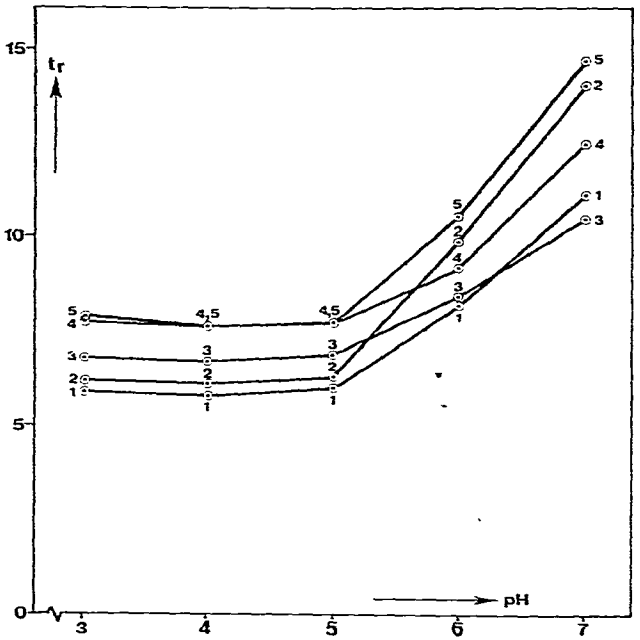


Fig. 1. Plot of the relative net retention, t_r, of the curare alkaloids against the pH of the mobile phase system. 1 = *d*-Tubocurarine chloride; 2 = chondrocurine; 3 = curarine chloride; 4 = isochondrodendrine; 5 = curine base.

In the latter instance, it is of interest that the two diastereoisomers chondrocurine (2) and curine (5) are eluted in close succession at the end of the chromatogram at pH 6 and 7, whereas they are eluted relatively far apart at lower pH values. Evidently the retardation of these two diastereoisomers is differently affected by the protonation of the two tertiary amino groups.

The decrease in retention of the double quaternary base curarine chloride (3) with decreasing pH can be explained by a decrease in the dissociation of the acidic silanol groups at lower pH values. Thus the retardation owing to the ion-exchange effect is mainly prevented.

A potassium phosphate buffer gave similar results. The optimal pH of the eluent seems to be 4 (see Fig. 1).

Nature of the cation

In recent investigations in our laboratory[21], it was found that the cation in the buffer is much more difficult than the anion to replace from the silica surface, owing to the acidic nature of the silica. In this connection, the effect of the nature of the cation on the reversed-phase separation of the curare alkaloids was also investigated, using 0.025 *M* potassium, ammonium and tetramethylammonium phosphate buffers in water–methanol.

The separations with potassium and ammonium phosphate buffer gave almost identical performances; tetramethylammonium phosphate buffer yielded a better separation. This is illustrated in Fig. 2, showing that with tetramethylammonium

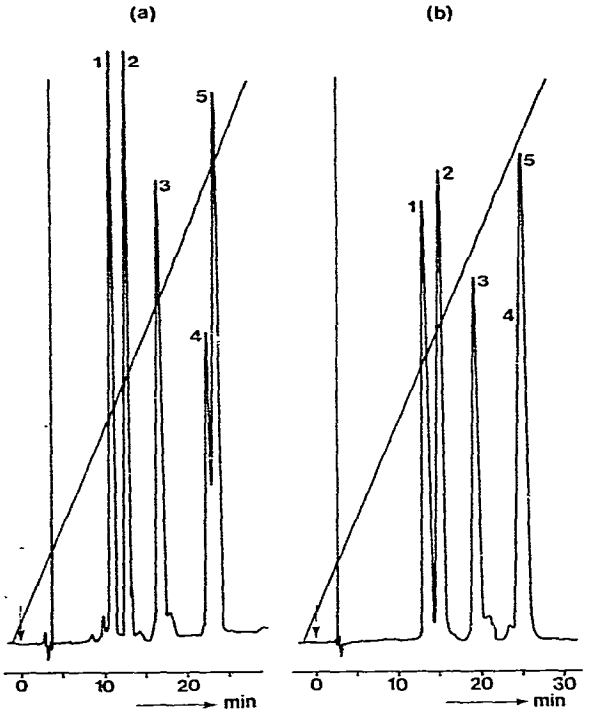


Fig. 2. Gradient HPLC separation of the test mixture of five curare alkaloids: (a) with 0.025 *M* tetramethylammonium phosphate buffer; (b) with 0.025 *M* potassium phosphate buffer. Alkaloids as in Fig. 1.

cation a better peak performance is obtained, together with an increased resolution; moreover the alkaloids are eluted faster with this buffer. At much higher concentrations a better peak performance can also be obtained with potassium and ammonium phosphate.

In view of these results and its better solubility in water–methanol, we decided to use tetramethylammonium phosphate.

Concentration of the buffer

Variation of the concentration of the tetramethylammonium phosphate buffer from 0.001 to 0.050 *M* at pH 4 had no influence upon the retention of the alkaloids; only the peak performance improved with increasing buffer concentration. Fig. 3 shows this effect.

At a concentration of 0.005 *M* of tetramethylammonium phosphate, no baseline separation was achieved between *d*-tubocurarine chloride (1) and chondrocurine (2), owing to the asymmetry of the peaks. With 0.020 *M* tetramethylammonium phosphate the peak performance was much better and baseline separation between (1) and (2) was obtained. With buffer concentrations above 0.020 *M*, no further improvement was achieved. A slight drawback to the higher buffer concentration is the relatively strong baseline drift compared with lower buffer concentrations.

The identical retention times at variable buffer concentrations again confirm

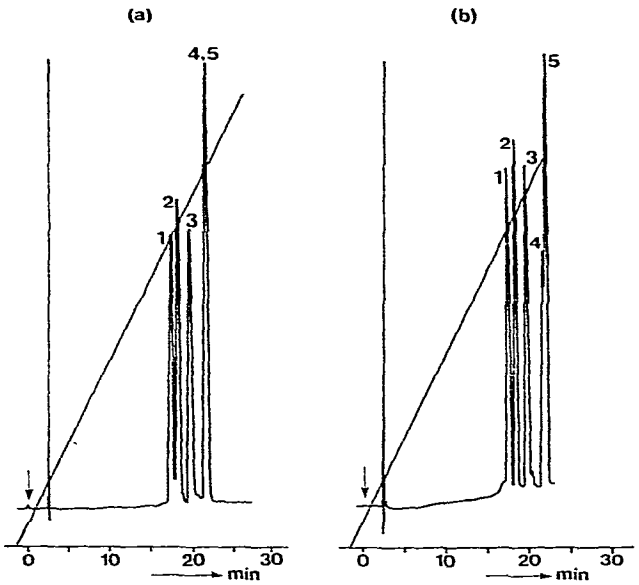


Fig. 3. Gradient HPLC separation of the test mixture of five curare alkaloids: (a) with 0.005 *M* tetramethylammonium; (b) with 0.030 *M* tetramethylammonium. Alkaloids as in Fig. 1.

that an ion-exchange effect has to be eliminated by decreasing the pH of the eluent to 4 in order to achieve the elution of the strong alkaloid bases from the RP-modified silica column. The application of a tetramethylammonium phosphate buffer serves only to further deactivate the undissociated silanol groups by which a better peak performance is obtained[21]. Further, it seems that a stable situation on the silica surface during the overall chromatographic procedure is obtained only if buffers with a sufficient capacity (concentration above 0.020 *M*) are used. At these high concentrations, the fluctuations of the tetramethylammonium concentration on the silica surface during the chromatographic run will be relatively small.

For the quantitative determination of *d*-tubocurarine chloride, a buffer concentration of 0.025 *M* was chosen.

Optimization of the gradient

Evaluation of all of the findings in this study resulted in a suitable chromatographic system for the separation of the curare alkaloids in the plant extract curare. In order to minimize undesirable mixing effects to a minimum, the water–methanol gradient was optimized in such a way that the two pumps deliver water–methanol mixtures that differ in composition as little as possible (75:25 and 55:45, respectively).

The application of isocratic elution was also investigated, but this procedure gave a considerable increase in the analysis time compared with gradient elution. A step gradient immediately after the elution of *d*-tubocurarine chloride led to about the same analysis time as in gradient elution. A step gradient, however, has several drawbacks with regard to gradient elution, *e.g.*, the peak performance is inferior, a much poorer separation of the other alkaloids is achieved, and the risk of column damage is increased.

Quantitative analysis

A pre-column was preferred to a clean-up procedure because the former method gives no losses of *d*-tubocurarine chloride and is much less laborious. At least 50 samples can be analysed before the pre-column has to be renewed.

The proportionality of peak area to the amount of *d*-tubocurarine chloride injected was measured in the range 1–9 μg. The calibration graph proved to be linear in this range.

Each run in the quantitative analysis of *d*-tubocurarine chloride in curare takes about 45 min (including regeneration of the column at the end of the gradient). Fig. 4 shows the chromatogram of a curare sample.

By repeated injection of the same curare solution, the coefficient of variation of the chromatographic procedure was determined to be 0.3% ($n = 6$) at a *d*-tubocurarine chloride level in the curare of 5%. The coefficient of variation for the whole analysis (including sampling and weighing) was 1.3% ($n = 6$).

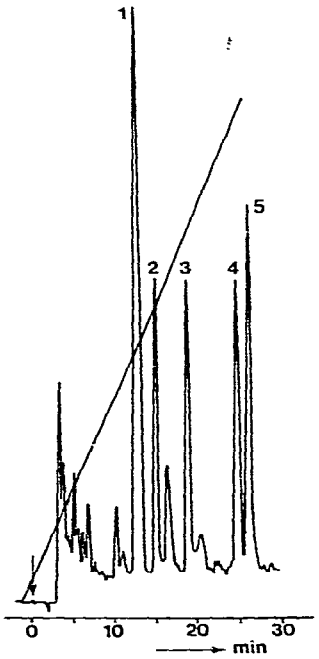


Fig. 4. Gradient HPLC chromatogram of a curare sample. Alkaloids as in Fig. 1. Injection: 20 μl of aqueous solution containing 28 μg of curare. *d*-Tubocurarine chloride peak corresponds to 1.42 μg or 5.07% calculated on curare.

CONCLUSIONS

(1) Quaternary ammonium bases such as the curare alkaloids can be analysed on a reversed-phase column.

(2) In order to prevent undesirable retardation effects (*e.g.*, ion exchange), an electrolyte has to be added to the mobile phase.

(3) The pH of the mobile phase affects both the retention and the retention sequence of the curare alkaloids.

(4) The nature of the cation in the buffer used affects both the retention and the peak performance; the concentration of the cation influences the peak performance.

(5) Both an accurate and rapid determination of *d*-tubocurarine in the plant extract curare is possible.

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