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CHROMATOGRAPHY OF BIS-QUATERNARY AMINO STEROIDS

I. SEPARATION ON SILICA BY THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Thin-layer (TLC) and high-performance liquid chromatographic (HPLC) methods have been developed for the separation of bis-quaternary amino steroids. The separations were performed on a silica layer and in a column, respectively. A mixture of methanol and acetonitrile was used as the organic solvents in the eluent, which contained concentrated ammonia, ammonium chloride and ammonium carbonate. In the HPLC experiments the effects of ammonium chloride and ammonium carbonate concentrations and the nature and concentration of the organic solvents in the eluents on the retentions of the compounds and separation efficiency were studied in detail.

The HPLC separation system could be transformed into a TLC system using an HPTLC silica layer and a slightly modified eluent mixture, resulting in a similarly effective separation of the compounds with the same elution order. The chromatogram was evaluated by densitometry after colour reaction of the separated spots.

INTRODUCTION

Quaternary amino steroids play a very important role in medicine and pharmacy. Pipecuronium bromide, a bis-quaternary amino steroid developed at the Chemical Works of Gedeon Richter (Budapest, Hungary) is used as a medium-acting neuromuscular blocking agent[1,2].

Only a few methods have been described for the separation and determination of quaternary amino steroids. Thin-layer chromatography (TLC) on silica[3,4] and alumina[5–7] layers has recently been applied to investigate the degradation of quaternary amino steroid-containing injectables.

The main aim of this work was to develop high-performance liquid chromatographic (HPLC) and TLC methods for the separation and determination of pipecuronium bromide and related steroids. The basic concepts of the separation system were devised by de Zeeuw and co-workers[4,8], who described the ion-pair adsorption

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chromatography of primary, secondary, tertiary and quaternary amines on a silica layer in which the basic drug cations migrated as uncharged ion pairs using inorganic anions as ion-pair formers.

In this paper, the optimization of the HPLC system with a silica stationary phase for the separation of bis-quaternary amino steroids and the possibility of the transformation of the optimal HPLC system to a TLC separation of the same components are considered.

EXPERIMENTAL

High-performance liquid chromatography

A Liquochrom 2010 high-pressure liquid chromatograph equipped with a variable-wavelength UV detector and a loop-type injector (Labor MIM, Esztergom-Budapest, Hungary) was used. The separations were performed on LiChrosorb SI 60 (10 μm) column (250 × 4.6 mm I.D.) (Chrompack, Middleburg, The Netherlands). The compounds were detected at a wavelength of 218 nm. The solvents used for the preparation of the eluents were of HPLC grade and were obtained from Merck (Darmstadt, F.R.G.).

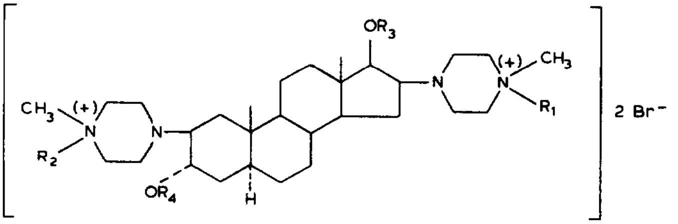
Thin-layer chromatography

The separations were performed on an HPTLC silica layer (Merck, Art No. 5629). The eluent was methanol–acetonitrile–concentrated ammonia solution (514:386:100) containing $5 \cdot 10^{-3}$ mole/dm^3 ammonium chloride and $8 \cdot 10^{-2}$ mole/dm^3 ammonium carbonate. The inorganic salts were dissolved in concentrated ammonia solution prior to preparation of the eluent.

The compounds investigated were dissolved in methanol and the solutions were applied to the plate in 1-cm strips. After spotting, the plate was placed in an unsaturated chamber and developed up to a distance of 5 cm. After the run, the plate was

TABLE I

STRUCTURES OF COMPOUNDS INVESTIGATED



Compound	R_1	R_2	R_3	R_4
I	H	H	CH_3CO	CH_3CO
II	CH_3	H	CH_3CO	CH_3CO
III	H	CH_3	CH_3CO	CH_3CO
IV	CH_3	CH_3	H	CH_3CO
V	CH_3	CH_3	H	H
VI (pipecuronium bromide)	CH_3	CH_3	CH_3CO	CH_3CO

dried in an air stream at room temperature and developed by immersion in Dragendorff reagent solution. The separated spots were evaluated by densitometry at 525 nm in the reflection mode.

Solvents and reagents

All reagents and solvents (except the HPLC-grade solvents) were of analytical-reagent grade and were obtained from Reanal (Budapest, Hungary).

The compounds investigated were prepared at the Chemical Works of Gedeon Richter and were of the highest available quality.

RESULTS AND DISCUSSION

The compounds investigated are listed in Table I. The model compounds can be divided into three groups: bis-quaternary compounds (IV, V and VI), mono-quaternary amino steroids (II and III) and a tertiary amino derivative (I). The polarity of the compounds depends on the number of quaternary amino groups and the presence or absence of ester groups.

Effect of ammonium chloride and ammonium carbonate in the eluent

Table II shows the change in the retention of the compounds investigated in the absence and presence of ammonium chloride and ammonium carbonate in the eluent.

When only ammonia solution is used in the eluent, no separation can be achieved. The retentions of the compounds are increased when a salt is added to the eluent. The best separation is obtained when both additives are used together, resulting in a better resolution and maximum column efficiency for pipecuronium bromide. Table II also shows a group-type separation of quaternary amino steroids.

TABLE II

EFFECT OF AMMONIUM CHLORIDE AND AMMONIUM CARBONATE IN THE ELUENT ON THE RETENTION OF THE SAMPLE COMPOUNDS

Conditions: column, LiChrosorb Si, 60, 10 μm (250 × 4.6 mm I.D.); eluent, mixture of A and B (88:12), where A is methanol–acetonitrile (1:1) and B is concentrated ammonia solution; flow-rate, 1 cm^3/min; detection at 218 nm. The resolution (R_s) was calculated for IV and VI and the plate height (H) was calculated for VI. Compounds: see Table I.

Compound	*Capacity factor (k')**			
	A	*B*	*C*	*D*
I	0.08	0.12	0.15	0.18
II	0.10	1.15	1.53	1.20
III	0.10	0.82	1.22	1.05
IV	0.25	4.95	9.40	6.30
V	0.25	7.25	14.3	9.35
VI	0.22	3.15	5.50	3.80
H (mm)	0.057	0.120	0.120	0.096
R_s	0.25	3.12	3.17	3.20

* A, Only concentrated NH_3; B, 0.1 mole/dm^3 NH_4Cl; C, 0.1 mole/dm^3 $(NH_4)_2CO_3$; D, 0.05 mole/dm^3 NH_4Cl + 0.05 mole/dm^3 $(NH_4)_2CO_3$.

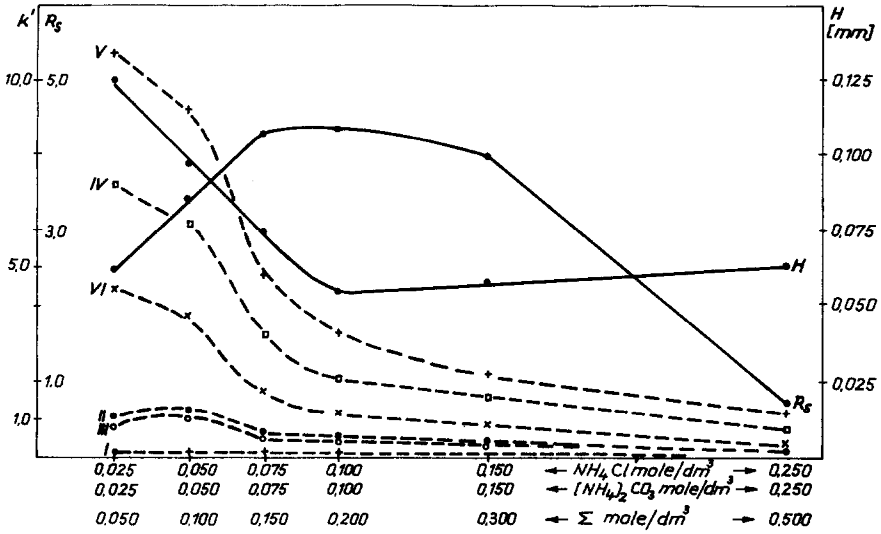


Fig. 1. Effect of salt concentration in the eluent on retention. Conditions as in Table II. Compounds: see Table I.

The bis-quaternary compounds are eluted before the mono-quaternary compounds and the tertiary amino derivative.

Effect of salt concentration in the eluent

The influence of salt concentration on the chromatographic separation is shown in Fig. 1. The retention of the compounds is considerably dependent on the salt concentration. An eluent containing 0.1 mole/dm^3 each of ammonium chloride and ammonium carbonate seems to be the most suitable.

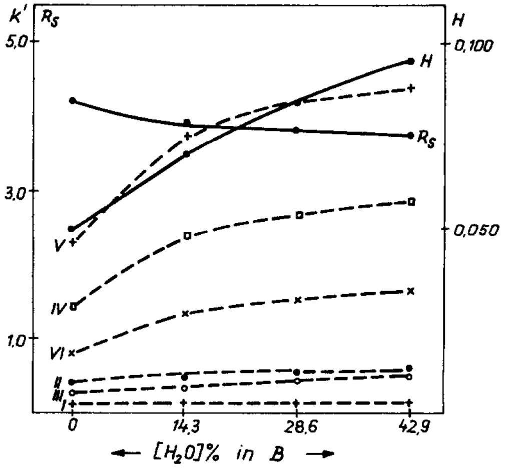
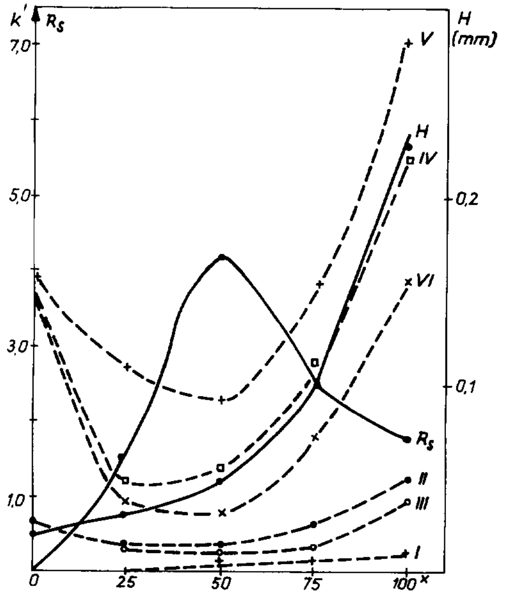


Fig. 2. Influence of water concentration in the eluent on the selectivity and efficiency of the separation. Conditions: eluent, mixture of A and B (86:14), where A is methanol–acetonitrile (1:1) and B is a mixture of concentrated ammonia solution and water containing ammonium chloride and ammonium carbonate each at a concentration of 0.1 mole/dm^3. Other conditions and compounds as in Table II.



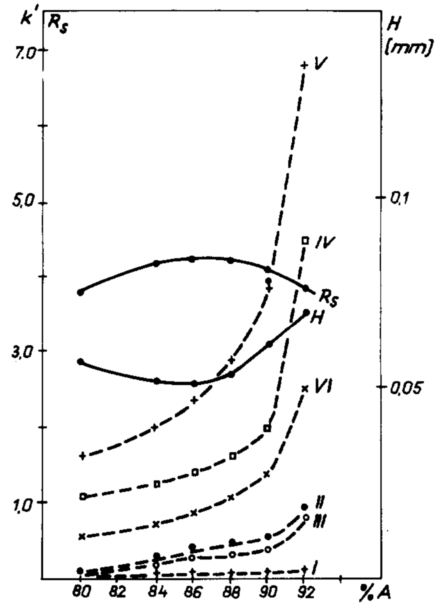


Fig. 3. Dependence of the separation on the proportions of methanol and acetonitrile. Conditions: eluent, mixture of A and B (86:14), where A is a mixture of methanol and acetonitrile in different proportions and B is concentrated ammonia solution containing 0.1 mole/dm^3 each of ammonium chloride and ammonium carbonate. Other conditions and compounds as in Table II. $x = 100 \cdot [CH_3OH]/[CH_3OH][CH_3CN]$.

Fig. 4. Influence of organic solvent concentration on the separation. Conditions as in Fig. 3; ratio of methanol to acetonitrile, 1:1.

Effect of water concentration in the eluent

As the separations performed on silica can be influenced by the concentration of water in the eluent, part of the eluent was replaced with water, using a constant organic solvent concentration in the eluent (Fig. 2).

No dramatic change in the separation characteristics was observed at low water concentrations, which is advantageous with respect to the reproducibility of the method. On increasing the water concentration the capacity ratios also increase, but the column efficiency decreases. The best separations were obtained when the contained no additional water.

Effect of nature and concentration of organic solvents on the selectivity and efficiency of the separation

Fig. 3 shows the dependence of capacity ratios, resolution and column efficiency on the proportions of methanol and acetonitrile in the eluent at a constant organic solvents concentration. It can be concluded that the best separation is achieved at 1:1 ratio of acetonitrile and methanol. At low methanol concentrations (acetonitrile-rich eluent) the separation is affected by the similar retentions of IV and VI, and at high methanol concentrations (acetonitrile-lean eluent) the column efficiency is not satisfactory for the separation. Maximum resolution is achieved at a 1:1 ratio of methanol and acetonitrile.

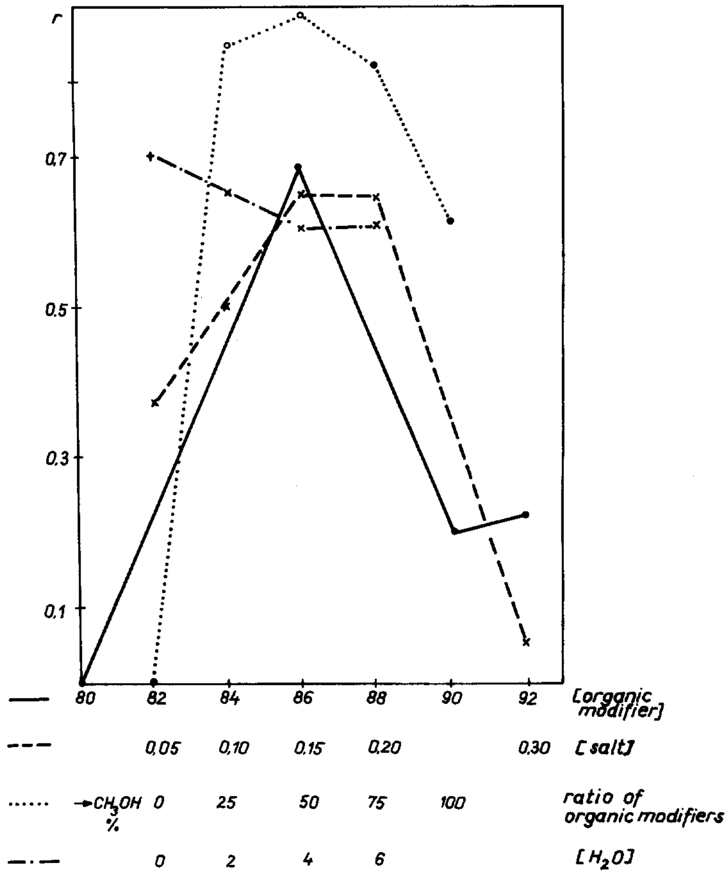


Fig. 5. Dependence of *r* values[9,10] on the chromatographic conditions.

Fig. 4 illustrates the influence of organic solvent concentration on the retention of the compounds at a ratio of methanol to acetonitrile of 1:1. The best separation is achieved at an organic solvent concentration of about 85%.

Summary of the optimization procedure in HPLC experiments

The resolutions shown in Figs. 1–4 were calculated for pipecuronium bromide (VI) and the next eluting compound (IV). For the control of the optimization procedure the *r* values were calculated from the individual R_s values according to De Galan and co-workers[9,10] and are shown in Fig. 5.

It can be concluded that the optimization procedure was correct; the best separations can be achieved on silica by using an eluent containing methanol and acetonitrile (1:1) as the organic solvent, at a concentration of (86%), and ammonium chloride and ammonium carbonate dissolved in concentrated ammonia solution in 0.1 mole/dm^3 concentrations each.

A model chromatogram is shown in Fig. 6.

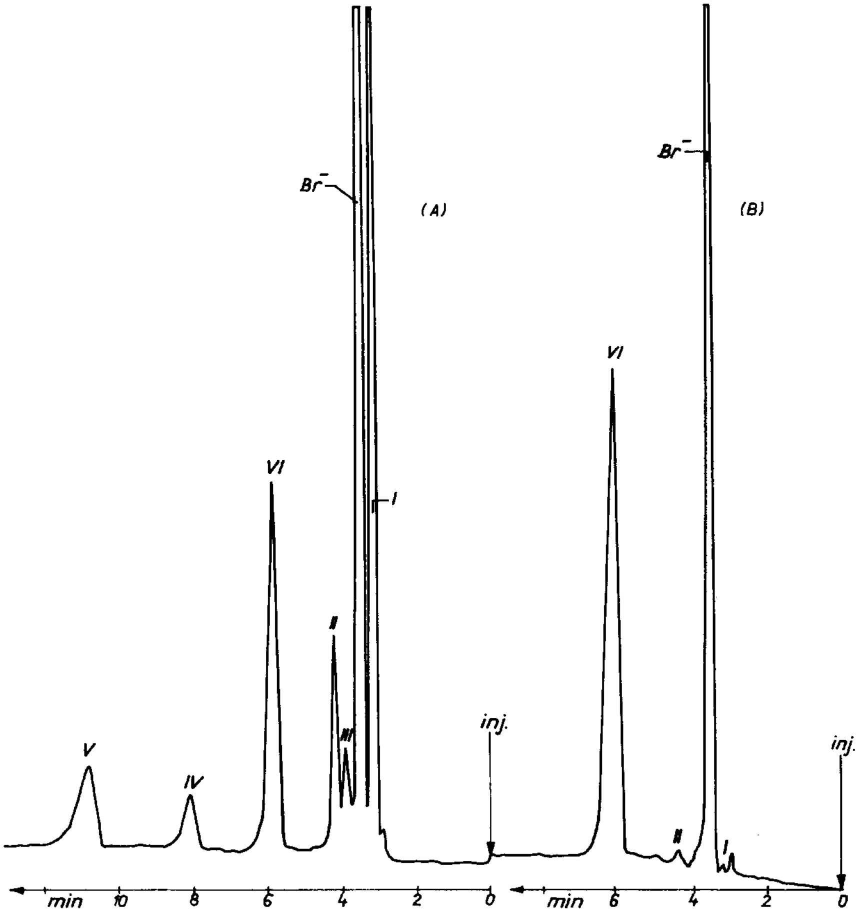


Fig. 6. Separation of pipecuronium bromide and related steroids by HPLC. Conditions as in Fig. 4; ratio of methanol to acetonitrile, 1:1. Compounds: see Table I. (A) Model chromatogram; (B) chromatogram of pipecuronium bromide raw material.

Transformation of the HPLC separation system to a TLC system

The eluent system used in the HPLC experiments can be used for TLC separation on an HPTLC silica layer. In the TLC experiments, considering the important role of the vapour phase during the separation, a slight change in the eluent composition was necessary.

Fig. 7 shows the densitogram obtained when the optimal TLC eluent was used. The elution order is the same in both the TLC and HPLC separations, and similar good resolutions for the compounds investigated were achieved.

CONCLUSION

In the desorbed HPLC and TLC methods, the retardation of the compounds investigated depends on the number of quaternary amino groups and on the presence

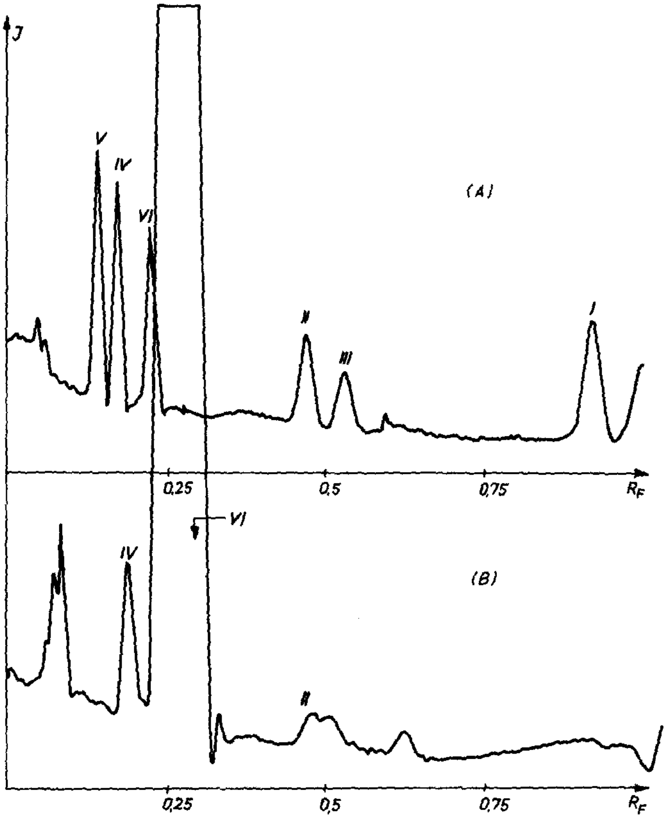


Fig. 7. Separation of pipecuronium bromide and related steroids by TLC. Conditions: see Experimental. (A) Model chromatogram; (B) chromatogram of pipecuronium bromide raw material. J = intensity of reflected light.

or absence of ester groups. In the HPLC separation, the tertiary amino derivative (I) is eluted first, the mono-quaternary compounds are less retarded than the bis-quaternary derivatives, and the retention of bis-quaternary compounds is highly influenced by the presence of an ester group, the hydrolysed decomposition products having longer retention times than pipecuronium bromide. The method seems to be applicable to stability assays.

The separation of bis-quaternary amino steroids on chemically bonded phases in both reversed-phase and normal-phase systems has also been investigated and the results obtained will be published in Part II.

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