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SEPARATION OF *CIS–CIS*, *CIS–TRANS* AND *TRANS–TRANS* ISOMERS OF (±)-ATRACURIUM BESYLATE AND *CIS* AND *TRANS* ISOMERS OF ITS MAJOR QUATERNARY DECOMPOSITION PRODUCTS AND RELATED IMPURITY BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation and determination of isomer ratios of *cis–cis*, *cis–trans* and *trans–trans* isomers of (±)-atracurium besylate and *cis* and *trans* isomers of its major quaternary decomposition products and related impurity using an octadecylsilica column and acetonitrile–phosphate buffer mobile phases were studied. The influence of the acetonitrile and buffer concentration and the pH of the mobile phase on the capacity factor (k'), selectivity (α), resolution (R_s) and peak symmetry factor (S) of the atracurium isomers was investigated. It was found that the acetonitrile concentration influenced α, whereas the buffer concentration and the pH of the mobile phase affected only k', S and R_s. Hydrophobic and silanophilic interactions were factors in the retention mechanism of the isomers under the conditions investigated.

INTRODUCTION

Atracurium besylate is a highly selective, competitive (non-depolarizing) neuromuscular blocking agent[1]. In a previous paper[2] the simultaneous determination of atracurium besylate and its major decomposition products and related impurities by reversed-phase high-performance liquid chromatography (RP-HPLC) was reported. Atracurium has four chiral centres at C-1 and N-2 in the two tetrahydropapaverine units (Fig. 1). Because of molecular symmetry, the sixteen isomers that are

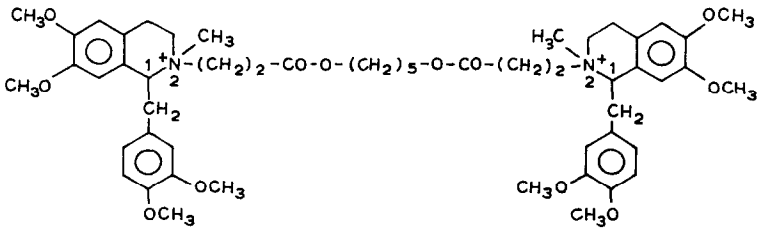

Fig. 1. Structure of atracurium.

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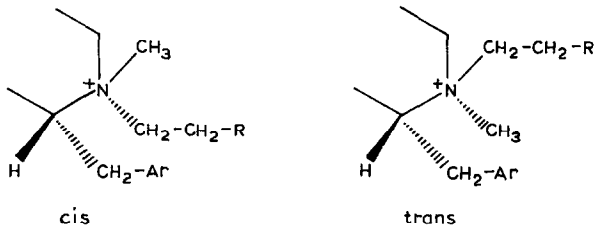

Fig. 2. Structures of *cis* and *trans* isomers.

theoretically possibly reduce to ten. These may be arbitrarily reduced by consideration of the relative configuration about the two C-1–N-2 bonds. The *cis* configuration is defined arbitrarily as that in which the two bulky substituents [1-(3,4-dimethoxylbenzyl) and 2-alkylene-ester groups] are *cis* (Fig. 2). Three configurations are possible for the molecule when considering the orientation of the groups at either end of the molecule, *cis–cis*, *cis–trans* and *trans–trans*. In the major decomposition products and related impurity of atracurium containing only one quaternary amino group, *cis* or *trans* configurations are possible. Stenlake *et al.*[3] had found that the atracurium isomers show different relative molar neuromuscular blocking potencies in anaesthetized cats. Therefore, analysis of atracurium for manufacturing and quality control purposes requires the ability to separate components similar in structure. HPLC with a silica stationary phase has been used to determine the total isomer content and *cis–cis*, *cis–trans* and *trans–trans* isomer ratios of atracurium[3,4]. The mobile phase used for the separation of the atracurium isomers contained hydrobromic acid and had a very low pH (below 2). This is a very aggressive mobile phase both for the silica stationary phase and the stainless-steel parts of the chromatographic system. The reported chromatographic conditions[3,4] can separate the *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium in about 10 min, but cannot separate the major decomposition products of atracurium from each other and the related impurity (monoquaternary analogue of atracurium; V) was not always clearly resolved from the peak of the *cis–cis* isomers of atracurium.

This paper describes an RP-HPLC method for the separation of the *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium and the *cis* and *trans* isomers of its major quaternary decomposition products and related impurity using isocratic and gradient elution.

EXPERIMENTAL

Chemicals

Atracurium besylate and its major decomposition products and related impurity were described in a previous paper[2]. The compounds studied for separating isomers are listed in Table I.

Acetonitrile (HPLC grade) and dibasic potassium phosphate trihydrate were obtained from Merck (Darmstadt, F.R.G.) and orthophosphoric acid from Fluka (Buchs, Switzerland).

TABLE I

COMPOUNDS STUDIED FOR THE SEPARATION OF STEREOISOMERS

No.	*Compound*
I	Atracurium
II	2-(2-Carboxyethyl)-2-methyl-1,2,3,4-tetrahydropapaverinium
III	2-(9-Hydroxy-3-oxo-4-oxanoyl)-2-methyl-1,2,3,4-tetrahydropapaverinium
IV	2-(3,11-Dioxo-4,10-dioxatridec-12-enyl)-2-methyl-1,2,3,4-tetrahydropapaverinium
V	2-Methyl-2,2'-(3,11-dioxo-4,10-dioxatridecamethylene)bis(1,2,3,4-tetrahydropapaverinium)

Equipment

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system consisting of two Model 501 pumps, a Model 440 ultraviolet detector equipped with a 280-nm filter and a 740 Data Module was employed. A Rheodyne (Berkeley, CA, U.S.A.) 7120 injection valve (10-μl sample loop) was used. A Nova-Pak C_{18} Radial-Pak cartridge (10 cm × 8 mm I.D.), particle size 5 μm, in a Radial Compression Module RCM-100 from Waters Assoc. and a Radelkis (Budapest, Hungary) Model OP-211/1 pH meter equipped with a glass electrode and a calomel reference electrode were used. The mobile phases and sample preparation were described in a previous paper[2].

The retention time of an unretained compound, t_0, was determined using sodium nitrate. The resolution of peaks, R_s, was calculated as

$$R_s = 2(t_2 - t_1)/(w_1 + w_2)$$

where t_1 and t_2 are the retention times and w_1 and w_2 are the base peak widths of the two peaks. Retention times were determined automatically by the integrator and peak widths at the base were measured manually from the integrator trace.

RESULTS AND DISCUSSION

The effect of mobile phase conditions (concentration of organic component and buffer and pH) on the capacity factor (k'), selectivity (α), R_s and peak symmetry factor (S) of the *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium was investigated. The peak symmetry factor was calculated as the ratio of the rear part to the front part of the peak at 10% of the peak height.

Effect of acetonitrile content in the mobile phase

Table II gives selected HPLC data for the three atracurium isomers to show the influence of the acetonitrile content in the mobile phase on k', α and R_s. A constant buffer concentration in the aqueous component of the mobile phase (0.1 mol/l) was maintained. It can be seen that a reduction in the percentage of the organic modifier in the mobile phase improves the isomer resolution. The α values in Table II show that they depend strongly on the acetonitrile content, but there is no significant difference in the influence of the acetonitrile content in the mobile phase on the column selectivity at different mobile phase pH (pH 5 and 3). With 28% acetonitrile in the mobile phase

TABLE II

EFFECT OF ACETONITRILE CONTENT IN THE MOBILE PHASE ON THE CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s) OF THE *CIS–CIS*, *CIS–TRANS* AND *TRANS–TRANS* ISOMERS OF ATRACURIUM AT DIFFERENT MOBILE PHASE pH AND 0.1 *M* PHOSPHATE BUFFER CONCENTRATION IN THE AQUEOUS COMPONENT OF THE MOBILE PHASE

The subscripts 1, 2 and 3 to k', α and R_s denote the *trans–trans*, *cis–trans* and *cis–cis* isomers of atracurium, respectively.

pH	*Acetonitrile (%)*	k'_1	k'_2	k'_3	α_{21}	α_{31}	α_{32}	$R_{s_{21}}$	$R_{s_{31}}$	$R_{s_{32}}$
5.0	28	10.06	11.44	12.56	1.13	1.25	1.10	2.09	3.48	1.29
	30	4.88	5.38	5.75	1.10	1.18	1.08	1.56	2.07	0.84
	35	1.94	2.06	2.19	1.06	1.11	1.05	0.86	1.54	0.64
	40	1.12	1.12	1.12	1.00	1.00	1.00	0.74	1.24	0.45
3.0	28	9.41	10.68	11.77	1.13	1.25	1.10	2.36	3.88	1.50
	35	1.81	2.00	2.12	1.10	1.14	1.06	1.08	1.79	0.80
	40	0.75	0.75	0.75	1.00	1.00	1.00	–	–	–

a good separation of the *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium for determining isomer ratios was obtained.

Effect of pH of the mobile phase

Fig. 3 shows the pH dependence of the peak symmetry factor S of the *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium when using 28% acetonitrile and 0.1 *M* phosphate buffer solution. It is clear that the peak symmetry is affected by the

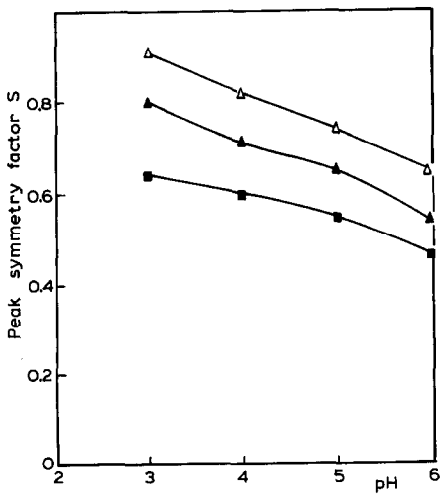

Fig. 3. pH depencence of the peak symmetry factor, S, of the (■) *cis–cis*, (▲) *cis–trans* and (△) *trans–trans* isomers of atracurium.

TABLE III

EFFECT OF MOBILE PHASE pH ON THE CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s) OF THE *CIS–CIS*, *CIS–TRANS* AND *TRANS–TRANS* ISOMERS OF ATRACURIUM AT 28% ACETONITRILE AND 0.1 *M* PHOSPHATE BUFFER CONCENTRATION IN THE AQUEOUS COMPONENT OF THE MOBILE PHASE

Subscripts 1, 2 and 3 as in Table II.

pH	k'_1	k'_2	k'_3	α_{21}	α_{31}	α_{32}	$R_{s_{21}}$	$R_{s_{31}}$	$R_{s_{32}}$
3	9.41	10.68	11.77	1.13	1.25	1.10	2.36	3.88	1.50
4	9.61	10.88	11.97	1.13	1.24	1.10	2.28	3.82	1.45
5	10.06	11.44	12.56	1.13	1.25	1.10	2.09	3.48	1.29
6	10.49	11.84	12.96	1.13	1.24	1.10	1.67	2.81	1.16

mobile phase pH. This effect can be explained by the dual retention mechanism[5] for the atracurium isomers and the other compounds investigated, in which both hydrophobic and silanophilic interactions on the surface of the reversed-phase octadecylsilica stationary phase dictate the chromatographic behaviour of solutes. The silanophilic interaction can include ionic interaction (ion exchange) between the quaternary amino groups or protonated amines and the dissociated surface silanol groups of octadecylsilica. Silanophilic interaction can have undesirable effects, for example excessive peak tailing (low S values). The cation-exchange behaviour of surface silanol groups in a chromatographic system will depend primarily on the pK_a of the silanols, the mobile phase pH, ionic strength and composition. The pK_a of surface silanol groups has been reported to be in the range 5–7[6]. At lower mobile phase pH the silanol group dissociation will be suppressed and the silanophilic interaction between the residual silanol groups and the amines decrease. This weaker silanophilic interaction leads to a better peak shape and a decrease in the k' values (Table III) of the atracurium isomers.

Table III illustrates that variation of the mobile phase pH over the range examined has no significant effect on the selectivity. It can be concluded that the increase in R_s values with decreasing mobile phase pH is caused by the better peak shape (higher S values) at lower mobile phase pH, *i.e.*, the column efficiency is improved.

TABLE IV

EFFECT OF BUFFER CONCENTRATION ON THE CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s) OF THE *CIS–CIS*, *CIS–TRANS* AND *TRANS–TRANS* ISOMERS OF ATRACURIUM AT 28% ACETONITRILE AND MOBILE PHASE pH 5.0

Subscripts 1, 2 and 3 as in Table II.

C_{buf} (*M*)	k'_1	k'_2	k'_3	α_{21}	α_{31}	α_{32}	$R_{s_{21}}$	$R_{s_{31}}$	$R_{s_{32}}$
0.025	11.50	13.25	14.69	1.15	1.28	1.11	1.83	3.00	1.10
0.050	11.12	12.50	13.75	1.12	1.24	1.10	1.87	3.23	1.25
0.100	10.06	11.44	12.56	1.13	1.25	1.10	2.09	3.48	1.29
0.150	7.88	9.00	9.76	1.14	1.24	1.08	1.80	2.73	1.09

Effect of buffer concentration in the mobile phase

Capacity factors were determined for the three atracurium isomers using mobile phases consisting of 28% acetonitrile and 72% aqueous solutions of potassium phosphate of concentration 0.025, 0.05, 0.1 and 0.15 mol/l. The mobile phase pH was maintained at 5.0.

The results are shown in Table IV. Over the range examined, k' decreased with increasing buffer concentration in the mobile phase. The α values in Table IV are not significantly affected by variations of the ionic strength of the mobile phase. The data in Table IV illustrate that the R_s values pass through a maximum at a buffer concentration about 0.1 mol/l.

Fig. 4 shows the effect of potassium phosphate concentration (C_{buf}) on the peak symmetry factor S. It can be seen that S increases with increasing buffer concentration in the mobile phase. The R_s values are affected by a combined effect of the variation of the buffer concentration in the mobile phase. With increasing buffer concentration in the mobile phase the peak shape is improved, which contributes to higher R_s values. On the other hand, the differences in the retention times of the isomers are decreased, which leads to lower R_s values. At buffer concentrations higher than 0.1 mol/l the second effect seems to be greater than the first, so that the R_s values effectively decrease. The potassium cations compete with the positivily charged solute for interaction on ion-exchange sites of the octadecylsilica[7,8]. The increase in the buffer concentration in the mobile phase will cause a decrease in the silanophilic interactions between the solute and the surface silanol groups of the stationary phase. Decreasing the silanophilic interactions will decrease the retention of the atracurium isomers (and the other compounds investigated) and contribute to increase the peak symmetry factors S.

Determination of isomer ratios

Fig. 5 shows a typical chromatogram for the determination of atracurium isomer

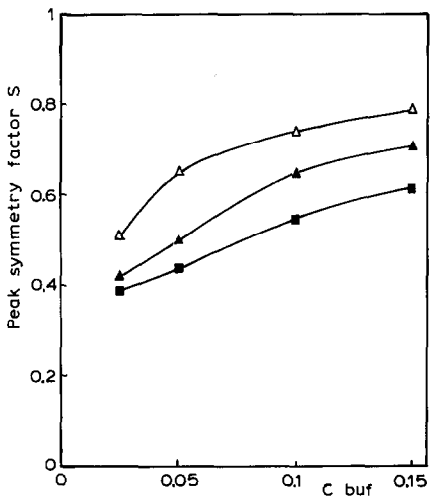

Fig. 4. Effect of buffer concentration on S values of *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium. Symbols as in Fig. 3.

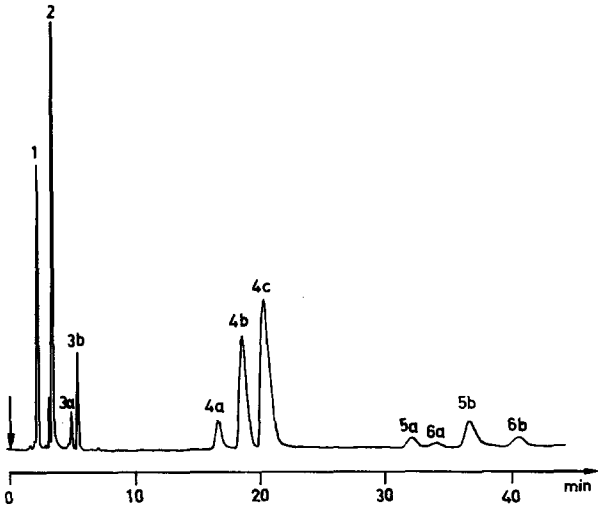

Fig. 5. Reversed-phase HPLC trace of partially decomposed (±)-atracurium besylate spiked with compound V showing the separation of the *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium. Column, Nova-Pak C_{18} Radial-Pak (10 cm × 8 mm I.D.; particle size, 5 μm). Mobile phase, acetonitrile–0.1 *M* potassium phosphate buffer (28:72), pH 5.0; flow-rate, 1.5 ml/min; detection, 280 nm (0.1 a.u.f.s.). Peaks: 1 = II; 2 = laudanosin; 3a, 3b = *trans* and *cis* isomers of III, respectively; 4a, 4b, 4c = *trans–trans*, *cis–trans* and *cis–cis* isomers of atracurium, respectively; 5a, 5b = *trans* and *cis* isomers of V, respectively; 6a, 6b = *trans* and *cis* isomers of IV, respectively.

ratios in the presence of its major decomposition products and related impurity using isocratic elution. It can be seen that it is possible to determine the isomer ratio of atracurium and its major quaternary decomposition products with the exception of compound II.

To determine the isomer ratio of compound II and the other compounds studied in the same run a gradient technique was applied. A representative chromatogram obtained by gradient elution of a mixture containing atracurium and its major decomposition products and related impurity is shown in Fig. 6. A mobile phase of pH 5.0 (about 5.5 in gradient elution) was chosen to avoid interference of the two peaks of compound V with those of atracurium or compound IV[2]. In the absence of compound V it is possible to use a mobile phase of pH 3 to improve the peak shape of the isomers.

As the pure *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium were not available, the identity of the peaks was deduced from literature results[4]. It is known[4] that the ratio of the *cis–cis*, *cis–trans* and *trans–trans* isomers of atracurium is approximately 10:6:1 (corresponding to about 59% *cis–cis*, 35% *cis–trans* and 6% *trans–trans* isomers, respectively). Because the *cis* isomer of the quaternary decomposition products of atracurium can be obtained from its *cis–cis* and *cis–trans* isomers, which show much higher percentages in atracurium than the *trans–trans* isomer, based on the overall *cis:trans* ratio in atracurium (approximately 3:1), the *cis* and *trans* isomers of the decomposition products should occur in a ratio of 3:1. This means that the larger of the two isomer peaks of a corresponding quaternary decomposition product of atracurium is associated with its *cis* isomers.

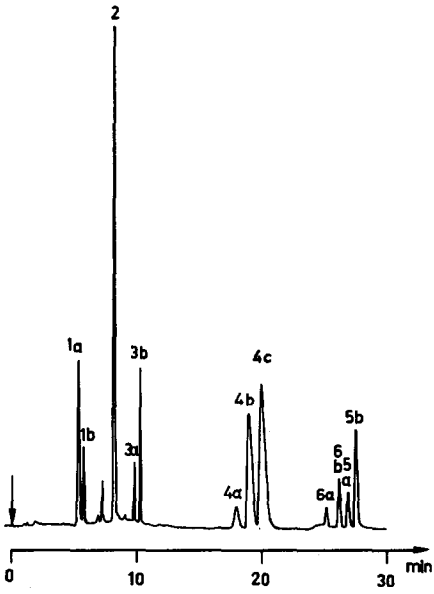

Fig. 6. Chromatogram obtained by gradient elution of partially decomposed (±)-atracurium besylate spiked with compound V (same sample as in Fig. 5). Column, Nova-Pak C_{18} Radial-Pak (10 cm × 8 mm I.D., particle size, 5 μm). Eluent: (A) acetonitrile; (B) acetonitrile–0.1 *M* phosphate buffer (1:10), pH 5.0; solvent programme, 2 min 7% A, 5 min linear gradient from 7% A to 21% A, 14 min isocratic at 21% A, 1 min linear gradient from 21% A to 30% A; flow-rate, 2.5 ml/min; detection, 280 nm (0.1 a.u.f.s.). Peaks: 1a, 1b = *cis* and *trans* isomers of II, respectively; other peaks as in Fig. 5.

For compound V the identities of the peaks were deduced by analogy with the results for atracurium (the larger peak should be associated with the *cis* isomer). In addition to these considerations, fractions containing the *cis–cis* isomer of atracurium were collected, which was degraded in acidic and basic media to obtain its quaternary decomposition products (*cis* isomers). Reinjection of these fractions containing the degraded *cis–cis* isomers of atracurium made possible the peak identification of the *cis* isomers of the quaternary decomposition products of atracurium based on retention times. For atracurium the *trans–trans* isomer is eluted before the *cis–trans* and *cis–cis* isomers. Comparision with literature results[4], for which silica (Partisil) was used as the stationary phase, illustrates the converse elution order of the atracurium isomers studied. For the quaternary decomposition products and related impurity of atracurium the *trans* isomer is eluted before the *cis* isomer, except for compound II, where the opposite occurs. It is difficult to explain this reversed retention order of compound II. The free carboxylic acid group of compound II and its smaller molecular size could make changes in the interactions between the isomers and the stationary and/or mobile phase possible. The results obtained in this work and those in ref. 4 indicate that hydrophobic and silanophilic interactions contribute to the retention of the compounds investigated.

Peak areas were measured and the isomer ratios were expressed as a percentage of the sum of the areas found for the three atracurium isomers. From the chromatograms showed in Figs. 5 and 6 the following results were obtained: 7.0%

trans–trans, 38.3% *cis–trans* and 54.7% *cis–cis* isomers with isocratic elution and 7.0% *trans–trans*, 38.0% *cis–trans* and 55.0% *cis–cis* isomers with gradient elution. The reproducibility (coefficient of variation; $n = 5$) of the isomer percentage for the same solution was 0.71% for the *trans–trans*, 0.50% for the *cis–trans* and 0.14% for the *cis–cis* isomer. By analogy with atracurium, the isomer percentage of its major quaternary decomposition products can be determined with similar reproducibility. From the chromatogram shown in Fig. 6 the following results for the *trans* isomers of the quaternary decomposition products and related impurity of atracurium were determined: 29.1% for II, 26.9% for III, 25.3% for IV and 24.4% for V. In this way it is possible to study the decomposition of atracurium with respect to its isomer rarios and that of the quaternary decomposition products obtained. The samples can also be successfully analysed on a LiChrosorb RP-18 column (25 cm × 4 mm I.D., particle size 5 μm) (Merck).

The results demonstrate that RP-HPLC is an efficient and rapid method for the simultaneous determination of isomer ratios of atracurium and its quaternary decomposition products and related impurity.

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