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Analysis of steroids

XLI[a]. Ion-pair high-performance liquid chromatographic separation of quaternary ammonium steroids on silica

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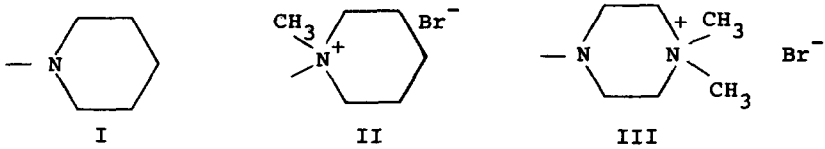
ABSTRACT

A normal-phase ion-pair chromatographic system has been developed for the high-performance liquid chromatographic investigation of pipecuronium bromide (2β,16β-bis-(N'-dimethyl-1-piperazinyl)-3α,17β-diacetoxy-5α-androstane dibromide) and related quaternary ammonium steroids. The use of silica as the stationary phase and a 96:4 mixture of acetonitrile and water containing 0.1 mol/dm^3 sodium perchlorate as the eluent with detection at 213 nm enable the potential impurities as well as the hydrolytic and oxidative degradation products of pipecuronium bromide to be separated and detected down to the 0.01% level. The above system is also applicable to the high-performance liquid chromatographic investigation of other quaternary ammonium steroids (pancuronium bromide, vecuronium bromide).

INTRODUCTION

Three steroidal neuromuscular blocking agents are currently used in therapy: pancuronium bromide (Pavulon®; Organon, Oss, Netherlands), vecuronium bromide (Norcuron®; Organon) and pipecuronium bromide (Arduan®; Richter, Budapest, Hungary). These are 2β,16β-disubstituted quaternary ammonium derivatives of 3α,17β-diacetoxy-5α-androstane. In the case of the bis-quaternary pancuronium bromide and pipecuronium bromide the substituents are II and III, respectively, while in the mono-quaternary vecuronium bromide the substituents at positions 2 and 16 are I and II (Fig. 1).



I II III

Fig. 1. Structures of the substituents of bis-quaternary pancuronium bromide (II) and pipecuronium bromide (III) and of the mono-quaternary vecuronium bromide at position 2 (I) and position 16 (II).

[a] For Part XL, see ref. 1.

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Only a few high-performance liquid chromatographic (HPLC) methods have been described in the literature for their separation and quantitative determination. Gazdag and co-workers [2,3] used silica as the stationary phase and a 43:43:14 mixture of acetonitrile, methanol and concentrated ammonia solution containing 0.1 *M* each of ammonium chloride and ammonium carbonate as the eluent for the batch analysis of pipecuronium bromide. This system enables the separation and quantification of pipecuronium bromide and its main impurities and degradation products [4]. A disadvantage is, however, the high ammonia and salt concentration of the eluent. The results obtained with this normal-phase hydrophobic interaction chromatographic system were superior to those obtained by the same authors [5] using various chemically bonded phases in reversed-phase hydrophobic interaction chromatographic systems.

For the determination of pancuronium bromide and vecuronium bromide in biological samples ion-pair chromatographic procedures have been described using iodide [6] or the highly fluorescent 9,10-dimethoxyantracene-2-sulphonate [7,8] as the ion-pairing reagents with pre- [6] or post-column [7,8] ion-pair extraction.

This paper describes a normal-phase HPLC system using neutral eluent containing sodium perchlorate as the ion-pairing reagent for the separation of quaternary ammonium steroids and their related impurities and degradation products.

EXPERIMENTAL

Apparatus

We used a Waters 600E multisolvent delivery system, a 990 photodiode array detector, a NEC/APC IV computer, a U6K variable-volume injector, a NEC CP6 pinwriter and a 990 plotter.

Chromatographic conditions

The column (250 × 4 mm I.D.) packed with SI 100, 5 μm, was purchased from Bio Separation Technologies (Budapest, Hungary). The UV detector was set to 213 nm. The separations were carried out at ambient temperature.

Various mixtures of acetonitrile and water (HPLC-grade purchased from Merck, Darmstadt, Germany) containing 0.1 mol/dm^3 sodium perchlorate (analytical reagent grade from Reanal, Budapest, Hungary) were used as the eluent at a flow-rate of 1 cm^3/min.

Test materials were injected in 20-μl samples of a 0.5% solution using the eluent as the solvent.

Samples

The three drugs were obtained from the manufacturers as listed in the introduction. The related steroids were synthesized by Dr. Z. Tuba (Chemical Works of Gedeon Richter).

RESULTS AND DISCUSSION

Aim of the study

The aim of the study described in this paper was to develop an HPLC system

suitable for purity testing of pipecuronium bromide bulk material, *i.e.* enabling the impurities and degradation products to be separated and quantified at the 0.01% level, and also for the assay of its formulations. It was also our aim that the eluent ensures the stability of both the sensitive ester-type test materials, the column and the HPLC equipment during the chromatographic run, *i.e.* that the use of alkaline eluents can be avoided.

The systems investigated were also checked for their applicability to the investigation of pancuronium and vecuronium bromides, but in these cases no detailed studies were carried out.

Selection of the ion-pair reagent

With their quaternary ammonium group(s) the test substances of this study are eminently suitable for the formation of ion pairs in neutral media with appropriate anions. This is the basis for a large variety of highly sensitive colorimetric and fluorimetric procedures for their determination mainly in biological samples [9] using various organic ion-pair forming agents. Many of them would also be suitable for the HPLC investigation with the great advantage of highly increasing the sensitivity of detection of the spectrophotometrically poorly active neuromuscular blocking agents [7,8]. To achieve this increase, however, an extraction step should be added to the procedure in order to separate the ion-pair complex from the bulk of the reagent. To avoid this we decided to select a simple inorganic anion: even the weak chromophores (tertiary and/or quaternary amino, as well as ester groups) provide sufficient sensitivity at short wavelengths to fulfil the aims of this study (ε_{215} of pipecuronium is 850).

Sodium perchlorate was selected as the ion pair-forming reagent. It has excellent spectral characteristics: it does not interfere with the estimation of the peaks of pipecuronium and related compounds. In addition, perchlorate anion is the strongest ion pair-forming inorganic anion: the logarithms of the extraction constants of the tetrabutylammonium ion pairs (organic phase: chloroform) in the order chloride, bromide, nitrate, iodide and perchlorate are -0.11, 1.29, 1.39, 3.01 and 3.48 [10].

A sodium perchlorate concentration of 0.1 mol/dm^3 was used throughout this study. The retention times of the investigated derivatives rapidly decrease with increasing concentrations of the ion pair-forming reagent up to about 0.05 mol/dm^3 and remain almost unchanged above 0.1 ml/dm^3, thus ensuring that the retention times will not be sensitive to minor changes in the reagent concentration around 0.1 *M*.

Optimization of the water/organic modifier ratio

The best results were obtained with the binary mixture of acetonitrile and water. The first important point to be taken into consideration is the relative position of the peak of the pipecuronium–perchlorate ion pair and that of the bromide ion, which is excluded from pipecuronium bromide by the stronger ion-pairing perchlorate ion and therefore migrates separately. The slopes of the increase of their capacity factors with increasing concentration of acetonitrile are different. Below about 70% of acetonitrile bromide is eluted first, then the elution order changes, but sufficient difference between their capacity factors can be obtained only at an acetonitrile concentration above 90%. This is important, as the majority of the potential impurities of pipecuronium bromide are eluted after the main peak and for this reason bromide should give the last peak if interference with the impurity peaks is intended to be avoided.

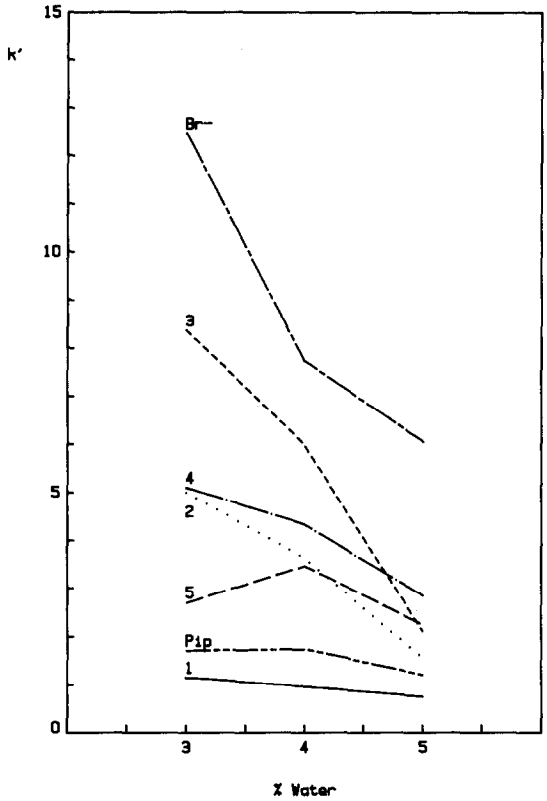


Fig. 2. Dependence of the capacity factor of pipecuronium (Pip) and its potential impurities on the water concentration in the eluent. For details, see Experimental.

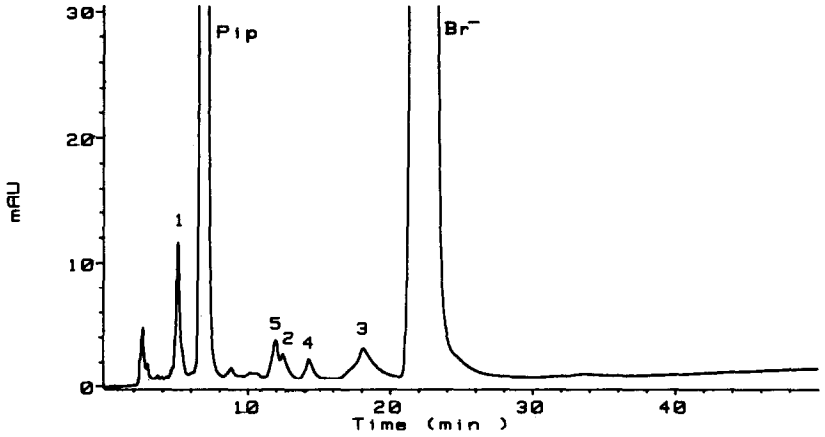


Fig. 3. Model chromatogram of pipecuronium bromide spiked with 0.1% of impurity 1 and 1% of each of impurities 2–5. Eluent, acetonitrile–water (96:4, v/v). For details see Experimental. The numbering of the impurities is summarized in Table I.

Fig. 2 shows that the critical range of water concentration is between 3 and 5% (v/v). An acetonitrile–water ratio of 96:4 has been selected for the separations.

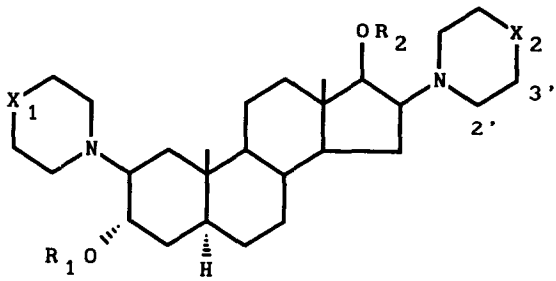
Advantageous features of the proposed system

Fig. 3 shows the chromatogram of pipecuronium bromide spiked with 1% quantitites of the potential impurities and decomposition products. The relative quantity of impurity 1 was 0.1% only because, as a consequence of its enamine structure, its absorptivity at 213 nm is about ten-fold that of pipecuronium bromide and the other impurities. (The limit of detection for impurities 2–5 and 1 was 10 and 1 ng, respectively.)

The structures of the impurites are shown in Table I. Impurity 1 is an oxidative decomposition product. Impurities 2 and 3 are isomeric monoquaternary derivatives (partially methylated products), while 4 and 5 are the isomeric monoacetyl derivatives (partially acetylated products or hydrolytic decomposition products). The good sep-

TABLE I

STRUCTURES OF PIPECURONIUM BROMIDE AND ITS IMPURITIES



Compound	R_1	R_2	X_1	X_2
Pipecuronium bromide (Pip)	Ac	Ac	$=N^+(CH_3)_2BR^-$	$=N^+(CH_3)_2Br^-$
1	Ac	Ac	$=N^+(CH_3)_2Br^-$	$=N^+(CH_3)_2Br^-$ 2'3'-dehydro
2	Ac	Ac	$=N\text{-}CH_3$	$=N^+(CH_3)_2Br^-$
3	Ac	Ac	$=N^+(CH_3)_2Br^-$	$=N\text{-}CH_3$
4	H	Ac	$=N^+(CH_3)_2Br^-$	$=N^+(CH_3)_2Br^-$
5	Ac	H	$=N^+(CH_3)_2Br^-$	$=N^+(CH_3)_2Br^-$

TABLE II

CHROMATOGRAPHIC DATA FOR QUATERNARY AMMONIUM STEROID DRUGS AFTER SEPARATION AS ION PAIRS WITH PERCHLORATE ION

See Fig. 2 for the chromatographic conditions.

Compound	k'	H (mm)	Asymmetry factor
Vecuronium	0.55	0.073	0.94
Pancuronium	0.61	0.079	0.88
Pipecuronium	1.60	0.079	0.95
Bromide	7.81	–	–

aration of the two pairs of isomers is a remarkable feature of the selectivity of the system. The poor resolution of impurites 2 and 5 does not cause problems in the course of using this method for the analysis of batches of pipecuronium bromide since in practice only impurity 5 occurs.

As regards our desire to introduce a non-corrosive system the results can be demonstrated by the following data. About 300 chromatographic runs were carried out over a period of 3 months using the same column and with no problems in the pump systems either.

Although no detailed studies were carried out with pancuronium bromide and vecuronium bromide, the data in Table II indicate that the described method seems to be suitable for their HPLC investigation too.

Further results concerning the validation of the quantification of the individual impurities will be the subject of another publication.

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