*Journal of Chromatography*, 421 (1987) 407–411
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3816

## Note

# Determination of ambenonium chloride in serum by reversed-phase ion-pair liquid chromatography

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(First received April 6th, 1987; revised manuscript received June 5th, 1987)

Ambenonium chloride is a quaternary ammonium compound possessing anticholinesterase properties. It is used for treating myastenia, a neuromuscular disorder, and is sold in France under the trade name Mytélase® (Winthrop). Its structure is given in Fig. 1.

Like neostigmine (Prostigmine®) and pyridostigmine (Mestinon®), ambenonium chloride contains quaternary ammonium function. The efficiencies of the antimyastenic effect of these three anticholinesterases appear to be comparable. However, the treatment is only symptomatic, and actually does not modify the evolution of the myastenia.

It is difficult to prescribe the most appropriate treatment based on subjective clinical improvement, because of risks of overdose [1–3]. Indeed, when using ambenonium chloride, an accurate adjustment of the amount and time of doses is required, as there is a rather low threshold between safe therapeutic amounts and overdoses leading to cholinergic intoxication, because cumulative effects can occur.

Until now, the fate of ambenonium chloride has remained completely unknown, since measurements in biological fluids are difficult. This is due to the extremely polar nature of ambenonium chloride, its large solubility in water, its low reactivity, its lack of volatility and the use of very small amounts in therapeutic applications.

Numerous methods of titrating quaternary ammonium compounds have been proposed, including ion-pair formation (by an anionic detergent or a dye) prior to titration, as well as spectrophotometric absorption in the visible and UV range [4,5], or indirect measurement by atomic absorption [6]. More recently, liquid

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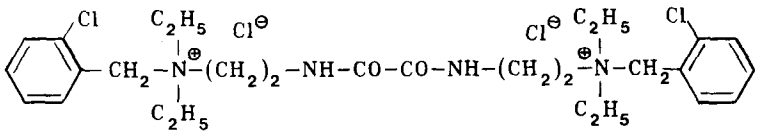

Fig. 1. Chemical structure of ambenonium chloride.

chromatography (LC) with ion-pair formation has been used in the aqueous phase titration of several quaternary ammonium compounds. High sensitivity could be achieved in some cases [7–10].

In this paper, we describe a reversed-phase ion-pair LC method for the measurement of ambenonium chloride by absorbance detection at 214 nm. An ion-pair extraction procedure, similar to the one of De Ruyter et al. [11], and involving picrate anion and tetrabutylammonium perchlorate as counter ions, is used to isolate the drug from biological material.

## EXPERIMENTAL

### *Chromatographic equipment*

The LC system consisted of a 110 A Beckman pump, a 160 absorbance detector (Beckman) with a Zn-lamp and a 214-nm filter, a 20-$\mu$l loop (Beckman) and a recorder (Kipp and Zonen, The Netherlands). The analyses were performed on a 25 cm$\times$4 mm I.D. stainless-steel column packed with 5-$\mu$m Nucleosil $C_{18}$ (Macherey-Nagel, Düren, F.R.G.). The mobile phase consisted of acetonitrile and aqueous 0.1 *M* sodium phosphate buffer (pH 3.5) containing 0.025 *M* lithium perchlorate (32:68, v/v). The mixture was filtered through a 0.45-$\mu$m filter. The flow-rate was set to 1 ml/min.

### *Chemicals and reagents*

Acetonitrile and dichloromethane were of liquid chromatographic grade (Carlo Erba, Milan, Italy). The distilled water used for all solutions and the mobile phase was purified through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Tetrabutylammonium perchlorate was purchased from Fluka (Buchs, Switzerland), picric acid from Aldrich (Milwaukee, WI, U.S.A.), sodium dihydrogenphosphate from Merck (Darmstadt, F.R.G.), lithium perchlorate from Frederick Smith (Columbus, OH, U.S.A.) and ambenonium chloride (Mytélase) from Winthrop (Clichy, France).

Tetrabutylammonium perchlorate was purified by dissolution in a minimal amount of chloroform and reprecipitation from diethyl ether.

### *Sample preparation*

To 1.0 ml of serum was added 0.5 ml of 0.1 *M* picric acid in 0.1 *M* sodium hydroxide (pH adjusted to 7); then 0.4 ml of 0.1 *M* sodium dihydrogenphosphate was added. The resulting mixture was extracted with water-saturated dichloromethane (12.0 ml) by shaking vigorously for 5 min. After centrifugation (2000

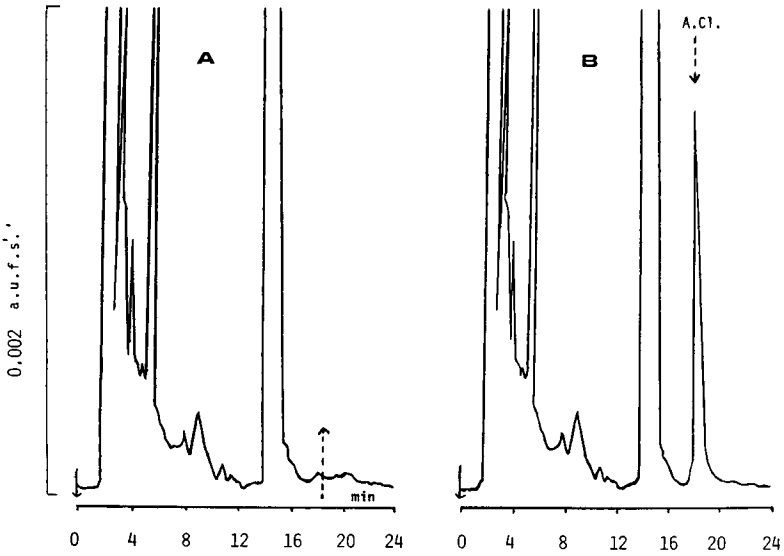


Fig. 2. Chromatograms of serum samples (A) without ambenonium chloride and (B) spiked with ambenonium chloride (0.4 μg/ml), at 0.002 a.u.f.s.

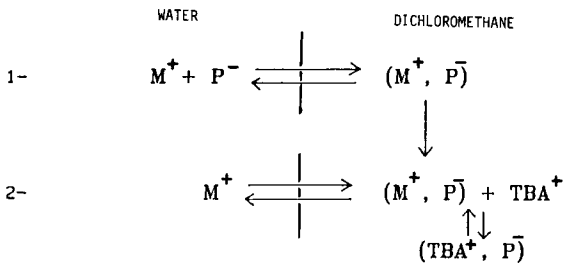


Fig. 3. Double extraction process in biological fluids. $M^+$ = Ambenonium cation; $P^-$ = picrate anion; $TBA^+$ = tetrabutylammonium cation.

*g*, 10 min), the aqueous phase, protein pellet and emulsified interface were removed with a Pasteur pipette.

The organic phase (10.0 ml) was then transferred to a PTFE-lined screw-capped conical centrifuge tube, and $10^{-3}$ *M* tetrabutylammonium perchlorate (200 μl) was added. After vigorous shaking for 30 s, the mixture was centrifuged (2000 *g*, 2 min) and 20 μl of the upper aqueous layer were injected into the LC system.

## RESULTS AND DISCUSSION

Under the conditions described, ambenonium chloride has a retention time of ca. 18 min. Fig. 2 shows chromatograms of a human plasma sample with ambenonium chloride and a human plasma blank. Peak heights are linearly related to the ambenonium chloride concentration over the 10–400 ng/ml range (correlation coefficient $r = 0.984$, $n = 6$). The standards were obtained by dissolving ambenonium chloride in a pool of plasma. To assess analytical recovery, we spiked

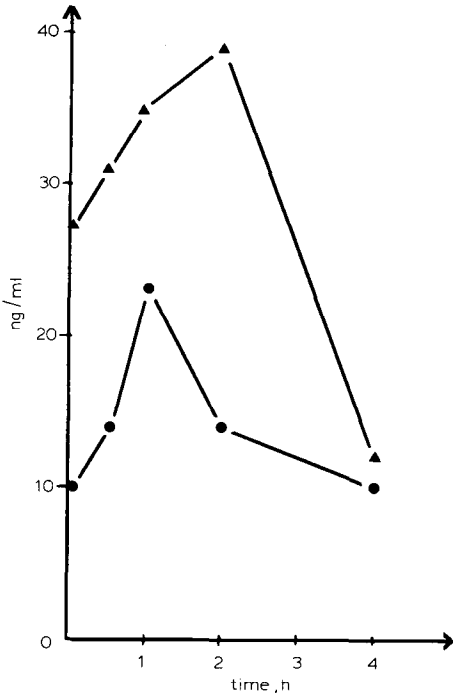


Fig. 4. Serum concentration curves of ambenonium chloride in two patients who received an oral dose of 5 mg (●) or 10 mg (▲) (chronic treatment).

human drug-free plasma with concentrations of 10, 50, 100, 200, 300 and 400 ng ambenonium chloride to 1 ml plasma. The coefficient of variation (C.V.) was 4.6% ($n=8$) at 200 ng/ml. We found a lower detection limit of ca. 10 ng/ml in human plasma.

The use of picrate anion to extract a variety of quaternary ammonium compounds is well known [12]. Ambenonium chloride forms an ion-pair, soluble in dichloromethane where picrate is the counter anion. Moreover, sodium picrate allows deproteinization.

In order to eliminate the picrate anion, which is responsible for an interfering peak on chromatograms, a second ion pair, more stable than the picrate–ambenonium pair, is formed between the tetrabutylammonium cation and the picrate anion. Then, the ambenonium cation returns to the aqueous phase (200 $\mu$l). This double extraction process in biological fluids is shown in Fig. 3. It results in higher purity and higher concentration.

Tetrabutylammonium ion is used as a $10^{-3}$ $M$ tetrabutylammonium perchlorate solution ($TBA^+P^-$).

Other quaternary ammonium compounds to be tested were tetrabutylammonium sulphate acid and tetrapentylammonium bromide. As purification of these turned out to be difficult, we adopted tetrabutylammonium perchlorate. Once purified, the salt leads to chromatograms free of any peak that would interfere with the ambenonium chloride peak. Use of tetramethylammonium chloride ($TBA^+Cl^-$) was also attempted, but the recovery of ambenonium chloride in the

final aqueous phase was inadequate: only ca. 30%. With $TBA^+P^-$, the recovery from serum was $75 \pm 2\%$ (200 ng/ml).

Other compounds with one or several quaternary ammonium functions were studied in order to define an internal standard, but none could be used satisfactorily. For this reason, each level is extracted in triplicate and analysed according to the procedure.

The application of the assay is demonstrated for two patients undergoing regular treatment who received an oral dose of 5 or 10 mg of ambenonium chloride (Fig. 4).

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