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REVERSED-PHASE, ION-PAIR LIQUID CHROMATOGRAPHY OF QUATERNARY AMMONIUM COMPOUNDS

DETERMINATION OF PYRIDOSTIGMINE, NEOSTIGMINE AND EDROPHONIUM IN BIOLOGICAL FLUIDS

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SUMMARY

A reversed-phase, ion-pair liquid chromatographic method for the quantitative determination of quaternary acetylcholinesterase inhibitors is described. The method uses an ion-pair extraction to isolate the drugs from biological material prior to liquid chromatographic separation and online UV detection at 214 nm. Quantitation down to 5 ng/ml and within-day precision with coefficient of variation (C.V.) of 1.5% (n=10, $\bar{x}$ = 100 ng/ml) for neostigmine, C.V., 1.7% (n=10, $\bar{x}$ = 80 ng/ml) for pyridostigmine and C.V., 1.5% (n=10, $\bar{x}$ = 100 ng/ml) for edrophonium have been achieved. The assay was designed for pharmacokinetic studies of these drugs in anesthetized patients.

INTRODUCTION

The acetylcholinesterase inhibitors pyridostigmine, neostigmine and edrophonium are used extensively in anesthesiology to reverse non-depolarizing neuromuscular blockade [1] and in the treatment of myastenia gravis, a neuromuscular disorder [2].

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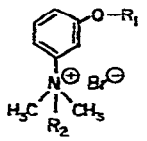
In order to study relationships between dose, serum level and effect, reliable quantitative methods to estimate the levels of these drugs are necessary. Methods available to quantitate these quaternary ammonium compounds include a spectrophotometric assay for pyridostigmine [3] and gas—liquid chromatographic assays for neostigmine and/or pyridostigmine [4—7]. The chromatographic assays depend on thermal dequaternization followed by thermionic detection [4,5] or selected ion monitoring [7] of the released tertiary amine or by electron-capture detection of the methyl iodide [6] formed in the process. Extraction of the drugs to be assayed is based on complexation with ion-pairing reagents [3—5] or with potassium triiodide [6]. Edrophonium, a reversible acetylcholinesterase inhibitor, can be assayed enzymatically [8]. These methods are tedious and/or limited in terms of sensitivity and reliability.



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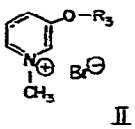

II

Ia : R_1 = —O—C(=O)—N(CH$_3$)$_2$; R_2 = —CH$_3$ Neostigmine bromide

Ib : R_1 = —H ; R_2 = —CH$_3$ 3-Hydroxytrimethylammonium bromide

Ic : R_1 = —H ; R_2 = —C$_2$H$_5$ Edrophonium bromide

IIa : R_3 = —O—C(=O)—N(CH$_3$)$_2$ Pyridostigmine bromide

IIb : R_3 = —H 3-Hydroxy-N-methylpyridinium bromide

This paper describes a reversed-phase, ion-pair liquid chromatographic method with absorption detection at 214 nm to quantitate the analytes and a modified ion-pair extraction procedure with picrate anion as counter ion for the isolation of the analytes from biological material. Retention behavior of pyridostigmine, neostigmine, their 3-hydroxy metabolites and edrophonium are examined. The assay was used to determine pharmacokinetic parameters of edrophonium, neostigmine and pyridostigmine. These results will be published in forthcoming papers.

EXPERIMENTAL

Chromatographic equipment

The liquid chromatograph consisted of a Varian 5020 pump (Varian Aerograph, Walnut Creek, CA, U.S.A.), an LDC 214-nm UV-III monitor Model 1203 with a Zn-lamp (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Valco CV-6-UHPA-N60 sampling valve with a 50-μl loop and a linear potentiometric recorder (Varian 9176).

Four reversed-phase columns were used in this study, two homemade and two from commercial sources. The homemade columns (15 × 0.32 cm) were packed with LiChrosorb RP-8 5 μm and LiChrosorb RP-18 10 μm (Merck, Darmstadt, G.F.R.) using a slurry technique. The commercial columns were

a Varian (30 × 0.4 cm) MCH 10-μm column and an Altex (15 × 0.46 cm) Ultrasphere Octyl 5-μm column (Altex, Berkeley, CA, U.S.A.). In order to avoid contamination of the analytical column, a pre-column (5 × 0.32 cm) tap-filled with Perisorb RP-2 (Merck, particle size 30—40 μm) was placed between the injector and the separation column.

Chemicals and reagents

Acetonitrile and dichloromethane were of liquid chromatographic purity (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). The water used for all solutions and mobile phases was doubly deionized. Heptanesulfonic acid sodium salt ($C_7H_{15}SO_3^-Na^+$) and tetramethylammonium chloride (TMA^+Cl^-) were obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Tetrabutylammonium hydrogen sulfate ($TBA^+HSO_4^-$) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium dihydrogen phosphate (NaH_2PO_4) and picric acid were Baker analyzed reagents (Baker Chemical, Phillipsburg, NJ, U.S.A.).

Crystalline neostigmine (I_a) and pyridostigmine (II_a) were obtained from Hoffman-LaRoche (Nutley, NJ, U.S.A.); edrophonium (I_c) was obtained from an intravenous injection solution (Tensilon® Hoffman-LaRoche). Hydrolysis in 2 *N* sodium hydroxide at 50°C for 4 h gave the corresponding 3-hydroxy metabolites (I_b = neostigmine metabolite; II_b = pyridostigmine metabolite) which were shown to be pure by liquid chromatographic analysis under conditions described for pyridostigmine and neostigmine (see Fig. 3). Neutralized solutions were used.

Chromatography

The influence of the following parameters on the chromatographic behavior of each quaternary ammonium compound was examined: TMA^+Cl^- concentration, $C_7H_{15}SO_3^-Na^+$ concentration, pH, acetonitrile concentration and support type.

Procedures for bioanalysis

Extraction from biological fluids. The internal standard used for the determination of pyridostigmine and neostigmine was edrophonium; neostigmine served as internal standard for the edrophonium assay. Standard solutions of these compounds (0.5 μg/ml) were prepared in water. To a PTFE-lined screw-cap culture tube (150 × 16 mm) were added 1.0 ml of serum, 100 μl of appropriate internal standard and 0.5 ml of 0.1 *M* picric acid in 0.1 *M* sodium hydroxide (pH adjusted to 7). Sodium dihydrogen phosphate (0.4 ml, 0.1 *M*) was then added. The resulting mixture was extracted with water-saturated dichloromethane (12.0 ml) by shaking vigorously for 5 min. Following centrifugation (2000 *g*, 10 min) the aqueous phase, protein pellet and emulsified interface were removed with the aid of a Pasteur pipet. The organic phase (10.0 ml) was then transferred to a PTFE-lined screw-capped conical centrifuge tube (134 × 17 mm) and 10^{-3} *M* tetrabutylammonium hydrogen sulfate (200 μl) was added. After vigorous shaking for 30 sec the mixture was centrifuged (2000 *g*, 2 min) and the majority of the lower organic phase was removed by means of a Pasteur pipet leaving approximate-

ly 400 μl. Care was exercised to avoid disturbing the aqueous layer. The remaining mixture was recentrifuged (2000 *g*, 1 min) and 50 μl of the aqueous phase were subjected to liquid chromatographic analysis.

Chromatography of biological extracts. The Altex 5-μm Ultrasphere Octyl column was used with the following mobile phases: for neostigmine and edrophonium assays, 0.01 *M* heptanesulfonic acid sodium salt, 0.01 *M* sodium dihydrogen phosphate, and 0.0025 *M* tetramethylammonium chloride in acetonitrile—water (20:80, v/v); for pyridostigmine assays, acetonitrile—water (17:83, v/v). The pH of the mobile phase was adjusted to 3 with concentrated sulphuric acid. The assays were performed at ambient temperature with a flow-rate of 2 ml/min. Detection was at 214 nm.

Quantitation. The procedure was standardized by analyzing drug-free serum samples spiked with known amounts of the analytes. Peak height ratios of analytes vs. internal standard were used to establish calibration curves. Serum concentrations in the unknown samples were determined using these calibration curves.

Recovery. Absolute overall recoveries from serum were estimated by comparison of peak heights obtained from the injection of known quantities of the analytes with peak heights obtained from the injection of extracts of serum samples spiked with the analytes.

Reproducibility. Within-day precision was determined by performing ten replicate analyses of spiked serum samples.

Treatment of glassware. The culture tubes used in the extraction were "conditioned" by storing them filled with a 0.1 *M* tetramethylammonium chloride solution. Before use the solution was poured off and the tubes rinsed five times with deionized water. The conical centrifuge tubes used in the back extraction were pretreated in the same way but were dried before use.

RESULTS AND DISCUSSION

Chromatographic behavior

Preliminary experiments revealed that pyridostigmine and neostigmine could not be eluted from the Varian MCH reversed-phase column (monomeric C_{18}, not end-capped) with acetonitrile—water mixtures unless TMA^+Cl^- was added to the mobile phase. These observations may be explained by the irreversible adsorption of the drugs to residual silanol groups and deactivation of adsorption sites by TMA^+. A similar observation was reported in the chromatography of curare alkaloids [9]. These investigators reported, however, that the concentration of TMA^+ did not affect retention volumes. We found that the capacity ratio of pyridostigmine and neostigmine could be regulated by altering the concentration of TMA^+ in the mobile phase (Fig. 1); increasing the concentration of TMA^+ decreased the retention volume. This was true for the MCH column employing a mobile phase free of ion-pair reagent and for all columns with heptanesulfonic acid in the mobile phase. This effect can be explained if the TMA^+ binds not only to the reactive silanol groups but also to the bonded stationary phase, resulting in a repulsion or a decreased availability of binding sites for pyridostigmine and

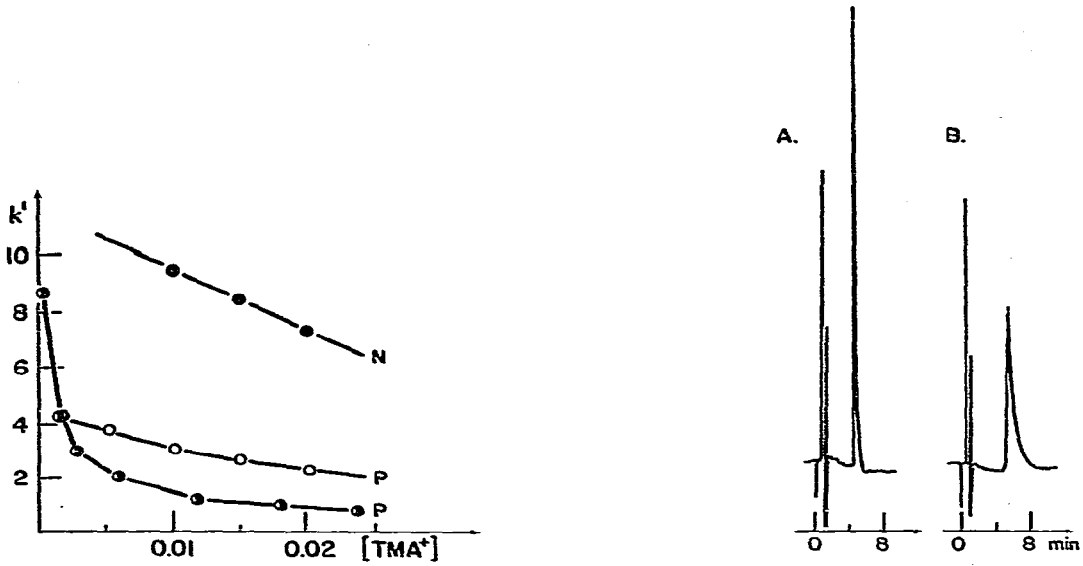

Fig. 1. Relationship between capacity factors (k') and tetramethylammonium (TMA^+) concentration. N = Neostigmine, P = pyridostigmine. ○ and ●: LiChrosorb RP-18 column with 0.005 *M* $C_7H_{15}SO_3^-Na^+$, 0.005 *M* NaH_2PO_4 and variable TMA^+ concentration in acetonitrile—water (15:85, v/v). ◉: MCH column with 0.01 *M* NaH_2PO_4 and variable TMA^+ concentration in acetonitrile—water (33:67, v/v).

Fig. 2. Comparison of peak shape from a 100-ng pyridostigmine injection (A) with 0.0025 *M* TMA^+ and (B) without TMA^+ in the mobile phase (0.01 *M* $C_7H_{15}SO_3^-Na^+$ and 0.01 *M* NaH_2PO_4 in acetonitrile—water (15:85, v/v). Column: 5 μm LiChrosorb RP-8 (15 × 0.32 cm); flow-rate, 1 ml/min.

neostigmine interaction. The addition of TMA^+ also had a pronounced effect on the peak shape. It reduced dramatically the tailing of the peaks obtained on the MCH 10-μm and the RP-8 5-μm column (Fig. 2). No effect on peak shape was noted for the RP-18 and the Ultrasphere Octyl columns.

The capacity ratios could be regulated further by changing the acetonitrile concentration and/or the ion-pairing reagent concentration (heptanesulfonic acid). A deviation from linearity with increasing concentration of the ion-pair reagent was observed and could have been due to the high concentrations used [10].

The effect of the pH was of dual nature. Not only was a decrease in retention volume observed at higher pH, but also a dramatic decrease in plate count. For neostigmine on the RP-18 column there were 600 plates at pH 5.7 vs. 2800 plates at pH 3.0.

The separation of pyridostigmine, neostigmine and their 3-hydroxymetabolites on the RP-18 column is demonstrated in Fig. 3.

We had some problems with the longevity of our home-packed columns. The RP-18 10-μm columns could be used for only two weeks before the plate count deteriorated. Also, the RP-8 5-μm columns developed a dramatic increase of back pressure after two days of use due to partial clogging of the bottom frit (2 μm) by fines. Therefore, we used an Altex Ultrasphere Octyl

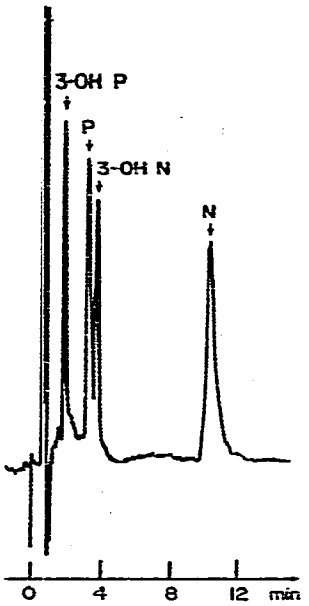

Fig. 3. Separation of neostigmine (N), pyridostigmine (P) and their 3-hydroxy metabolites (3-OH N, 3-OH P) on the 10-μm LiChrosorb RP-18 column. Mobile phase: 0.01 *M* $C_7H_{15}SO_3^-Na^+$ and 0.01 *M* NaH_2PO_4 in acetonitrile—water (15:85, v/v) adjusted to pH 3.0.

5-μm column for the work with biological materials. This column proved to be very stable and also had very good height equivalent to a theoretical plate (HETP) values for our samples. A value of 11.5 μm for neostigmine at a flow-rate of 1 ml/min without pre-column was achieved (15 μm with pre-column). To detect the low levels of compounds present in serum during our pharmacokinetic studies, a fixed-wavelength 214-nm detector was used. Neostigmine absorbs weakly at 254 nm ($\epsilon < 400$) compared with its absorption at 214 nm ($\epsilon > 6000$). Higher sensitivities for pyridostigmine also could be achieved at this low wavelength ($\epsilon_{254nm} < 2500$, $\epsilon_{214nm} > 7000$).

Bioanalysis

Iodide ion [4,5], dipicrylamine [3] and tetraphenylborate [11] have been reported as ion-pairing reagents for the extraction of neostigmine and pyridostigmine. Extraction recoveries with iodide were only 30% compared with the near-quantitative recoveries with the two other reagents. The use of picrate anion to extract a variety of quaternary ammonium compounds is well documented [12]. Due to its ready availability and utility over a broad pH range we examined picrate anion as a counter ion for biological extractions. To remove the picrate anion, which gives an interfering peak on the liquid chromatographic tracings, and to obtain cleaner extracts, a back-extraction step with 0.001 *M* TBA^+ HSO_4^- was employed. The TBA^+ helps to partition the analytes into the aqueous phase and keeps the picrate anion into the organic phase. Using untreated glassware, analysis of spiked (100 ng/ml) serum or aqueous samples afforded low recoveries and irrepro-

ducible results. Recoveries ranged from 42 to 78% for neostigmine and from 56 to 85% for pyridostigmine. Substitution of picrate anion with dipicrylamine at pH 9 did not improve recoveries. These low and variable recoveries can be explained by losses due to adsorption to glassware and/or interphase. Treatment of the glassware to deactivate adsorption sites could minimize this effect. As silanization was reported ineffective [12], another approach was used. Deactivation with TMA^+Cl^-, as described in the experimental section, increased extraction reproducibility and recovery (Table I) to a satisfactory level. Variation of extraction pH (4 to 9) had no pronounced effect on extraction behavior.

TABLE I

EXTRACTION RECOVERY

Substance	$\bar{x}$ (%)	S.D. (%)	C.V. (%)	n	Concentration (ng/ml)
Pyridostigmine	96.4	3.8	3.9	9	80
Neostigmine	99.1	2.4	2.5	9	100
Edrophonium	87.8	2.7	3.1	9	100

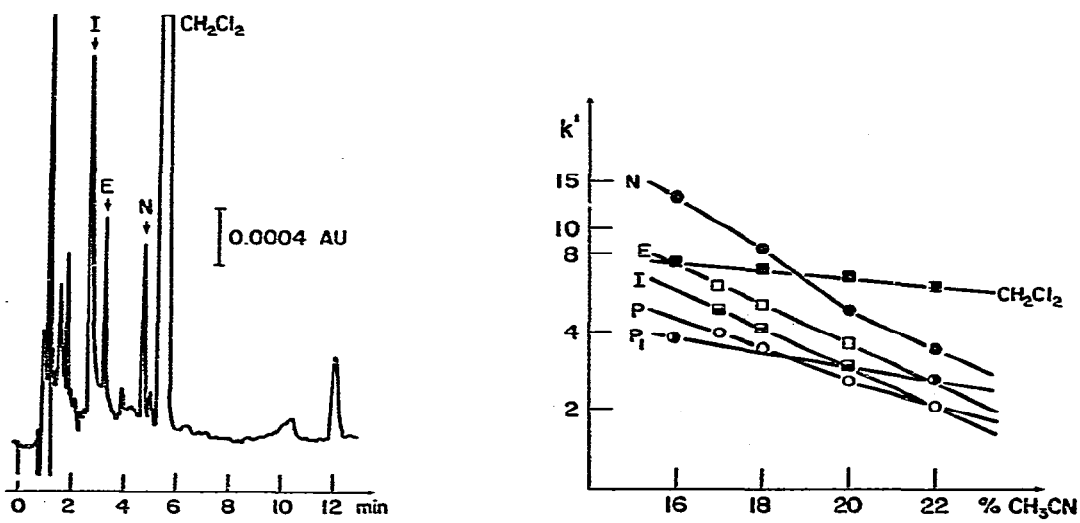

Fig. 4. Liquid chromatographic trace from the injection of an extract from a serum sample containing 53 ng/ml neostigmine. Eluent: 0.01 *M* $C_7H_{15}SO_3^-Na^+$, 0.01 *M* NaH_2PO_4 and 0.0025 *M* TMA^+Cl^- in acetonitrile—water (20:80, v/v), pH 3.0. Column: 5-μm Ultrasphere Octyl (15 × 0.46 cm). Flow-rate, 2 ml/min. Detection at 214 nm 0.004 a.u.f.s. Peaks: I = interference, E = edrophonium (internal standard), N = neostigmine and CH_2Cl_2 = dichloromethane present in the injection solution.

Fig. 5. Separation possibilities of analytes (N, neostigmine; P, pyridostigmine; E, edrophonium) and interference (I, CH_2Cl_2, Pi = picrate). Relationship between capacity ratios (k') and acetonitrile content of mobile phase. Column: 5-μm Ultrasphere Octyl (15 × 0.46 cm); eluent: 0.01 *M* $C_7H_{15}SO_3^-Na^+$, 0.01 *M* NaH_2PO_4 and 0.0025 *M* TMA^+Cl^- in acetonitrile—water adjusted to pH 3.0.

Peak height ratios and concentration were linearly related over the range of 0—400 ng/ml for neostigmine, 0—1000 ng/ml for pyridostigmine and 0—1500 ng/ml for edrophonium. The lowest points on the calibration graphs were 10 ng/ml for neostigmine, 30 ng/ml for pyridostigmine and 10 ng/ml for edrophonium. Quantitation down to 5 ng/ml (signal-to-noise ratio > 4) was achievable for all analytes.

Within-day precision of the assay was coefficient of variation (C.V.) 1.5% (n=10, $\bar{x}$ = 100 ng/ml) for neostigmine, C.V. 1.7% (n=10, $\bar{x}$ = 80 ng/ml) for pyridostigmine and C.V. 1.5% (n=10, $\bar{x}$ = 100 ng/ml) for edrophonium.

Chromatography of biological extracts showed several peaks on the liquid chromatographic tracing other than those from the analytes (Fig. 4). The dichloromethane dissolved in the aqueous back-extraction phase gave rise to a major peak (CH_2Cl_2 in Fig. 4). A large peak interfered with pyridostigmine but was not always present (I in Fig. 4). No interferences were found at the elution volumes of neostigmine and edrophonium. There was a selectivity difference for the dichloromethane peak and the large interference compared to the analytes. The concentration of acetonitrile in the mobile phase could be used to regulate the separation of analytes from these interfering peaks

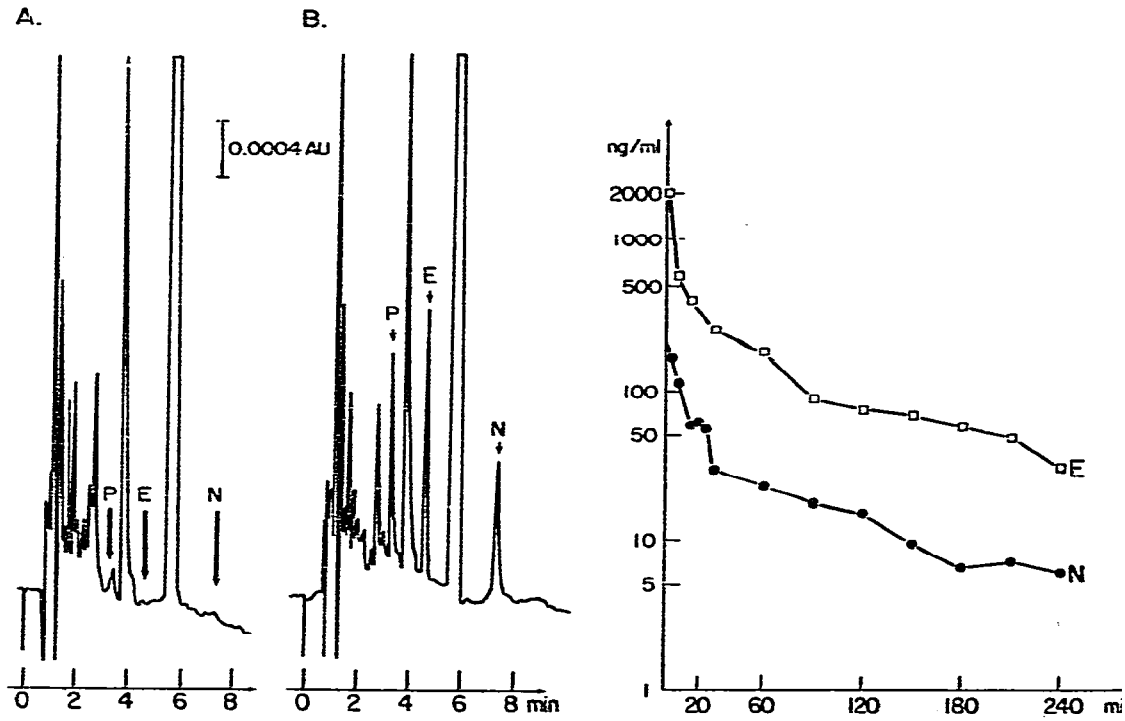

Fig. 6. Liquid chromatographic trace of extracts from blank (A) and spiked (B) serum samples using acetonitrile—water (17:83, v/v) in the mobile phase. Other conditions as in Fig. 4.

Fig. 7. Concentration versus time curves for neostigmine (N), and edrophonium (E) after intravenous administration. Infusion, 2 min; 0.5 mg/kg for edrophonium and 0.05 mg/kg for neostigmine.

(Fig.5). Use of 20% acetonitrile for neostigmine and edrophonium assays (Fig.4) and 17% for pyridostigmine assays (Fig.6) provided the optimum resolution. No attempts were made in this study to optimize parameters for the detection of the 3-hydroxy metabolites of pyridostigmine and neostigmine in biological fluids.

The procedure was employed to examine the pharmacokinetics of neostigmine, pyridostigmine and edrophonium. Examples of concentration versus time curves for neostigmine and edrophonium are shown in Fig.7. Serum concentrations could be followed up to 4 h after administration for neostigmine and beyond for edrophonium. The procedure also may be used to detect these drugs in urine.

CONCLUSION

The proposed method provides several advantages over methods currently available. An improved extraction procedure is used that gives better efficiencies and greater reproducibility. The method does not rely on thermal dequaternization processes that are difficult to control. It is a versatile method that can be used to analyze edrophonium and urine samples as well. Minor modifications would allow analysis of the 3-hydroxy metabolite of neostigmine. The method has the potential to monitor drug levels of pyridostigmine and neostigmine in myasthenia gravis patients.

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