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A QUANTITATIVE GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF NEOSTIGMINE AND PYRIDOSTIGMINE IN HUMAN PLASMA

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SUMMARY

A sensitive and selective analytical method was used to measure the concentration of neostigmine and pyridostigmine in human plasma. The procedure involved preliminary ion-pair extraction of the drugs into dichloromethane, followed by concentration and analysis of the ion-pair complex using a gas–liquid chromatographic system fitted with a nitrogen detector. Using the peak area ratio technique, pyridostigmine bromide was used as the internal standard for the quantitation of neostigmine in plasma; neostigmine bromide was the internal marker for the determination of pyridostigmine. The method depends on the thermal dequaternisation of the quaternary amines, and can be used to detect 5 ng/ml in a 3-ml plasma sample. Accurate measurement can be made at levels of 50–1000 ng/ml. This assay procedure has been applied to the separate determination of the plasma concentration of neostigmine and pyridostigmine after single administration of intravenous doses in aneasthetised patients.

INTRODUCTION

Quaternary amines that inhibit cholinesterase (for instance, neostigmine and its analogue pyridostigmine) have been widely used in the treatment of myasthenia gravis for over forty years[1,2]. In patients with this rare disorder of neuromuscular function, direct measurement of the plasma concentration of neostigmine or pyridostigmine might be extremely useful in the assessment of anticholinesterase therapy. Most methods previously used for the determination of quaternary amines in biological fluids have been based on their extraction as an ion-pair using a variety of counter ions[3–6]. The ion-pair is subsequently dissociated with dilute hydrochloric acid, and the quaternary amine is assayed by standard analytical techniques. These methods have been applied to the measurement of pyridostigmine in plasma[7]; thus, the drug can be extracted as an iodide complex into methylene dichloride and its absorbance measured at 293, 329 and 365 nm. The limit of sensitivity of the assay is 0.1–0.2 μg/

ml and interference by other drugs may occur[7]. We have had limited success with this procedure, which does not appear to separate pyridostigmine from its main metabolite[8,9]. The clearance of pyridostigmine from plasma and its excretion in urine have been studied in myasthenic patients by radioisotopic techniques[8], although these investigations are expensive and involve the administration of large doses of radioactivity.

Reliable methods for the determination of neostigmine in plasma have not been developed. Reversible inhibitors of cholinesterase (for instance, edrophonium chloride) can be assayed by enzymatic procedures[10], and these methods have been used to study the correlation between the plasma concentration of the drug and its pharmacological effects. Although these assay procedures are very sensitive, they are time-consuming and exacting.

Methods based on gas–liquid chromatographic (GLC) techniques have been used for the quantitative determination of quaternary amines in plasma[5,11,12]. Nevertheless, there is no published GLC method that will measure unchanged neostigmine or pyridostigmine in human biological fluids.

EXPERIMENTAL

Materials

The following materials were used: dichloromethane, diethyl ether and methanol, all of AnalaR quality and freshly redistilled; AnalaR anhydrous sodium sulphate; ether-washed sodium hydroxide solutions (5 *N* and 0.1 *N*); 0.1 *M* glycine buffer[13] (AnalaR glycine, 7.505 g, AnalaR sodium chloride, 5.850 g, and distilled water to 1 l); potassium iodide–glycine buffer (AnalaR potassium iodide, 12.8 g, 0.1 *N* sodium hydroxide solution, 4 ml, and 0.1 *M* glycine buffer, 6 ml); neostigmine bromide, U.S.P. (Koch-Light, Colnbrook, Great Britain); pyridostigmine bromide, U.S.P.; dimethylcarbamate ester of 3-dimethylaminophenol; dimethylcarbamate ester of 3-hydroxypyridine; edrophonium chloride (Roche, Welwyn Garden City, Great Britain); glycopyrronium bromide U.S.N.F. (A. H. Robins, Horsham, Great Britain); isopropamide iodide (Smith, Kline & French, Welwyn Garden City, Great Britain); mepenzolate bromide, U.S.N.F. (M.C.P. Pharmaceuticals, Livingston, Great Britain); penthienate methobromide (Sterling-Winthrop, Newcastle-upon-Tyne, Great Britain); [^{14}C]neostigmine iodide; [^{14}C]pyridostigmine iodide (The Radiochemical Centre, Amersham, Great Britain).

Apparatus

The following apparatus was used: 10-ml capacity centrifuge tubes with well-fitting screw caps (Sovirel, Levallois-Perret, France); 15-ml stoppered evaporating tubes with finely tapered bases as described by Beckett[14]; 10-μl S.G.E. microsyringe (Chromatography Services, Wirral, Great Britain); Perkin-Elmer Model 17 gas chromatography all-glass system, fitted with a phosphorus–nitrogen detector and linked to a Perkin-Elmer chart recorder Model 56 (Perkin-Elmer, Beaconsfield, Great Britain) and to an Autolab (Mountain View, U.S.A.) digital integrator Model 6300; coiled glass columns $\frac{1}{4}$ in. O.D.; gas–liquid chromatography–mass spectrometer system (Pye 104 gas chromatograph coupled to an MS 12 mass spectrometer; AEI,

Manchester, Great Britain); Pipetman microsyringes (Anachem, Bedford, Great Britain).

Gas chromatography

Methanolic solutions of neostigmine bromide, pyridostigmine bromide and other compounds were injected on to the following chromatographic systems at various oven temperatures:

(1) Diatomite CQ (100–120 mesh) coated with 3% (w/w) OV-1, 2 m × ¼ in. O.D. glass;

(2) Diatomite CQ (80–100 mesh) coated with 3.8% (w/w) SE-30, 2 m × ¼ in. O.D. glass;

(3) Diatomite CQ (100–120 mesh) coated with 3% (w/w) OV-17, 2 m × ¼ in. O.D. glass.

All columns were conditioned at 20° below the maximum recommended temperature of the relevant stationary phases for 24 h. Each column was then silanized twice *in situ* with 10-μl samples of hexamethyldisilazane before use.

Retention time, resolution and symmetry factors of the chromatographic peaks for these systems were then established.

Compounds that were investigated as possible internal markers were: edrophonium chloride, glycopyrronium bromide, isopropamide iodide, mepenzolate bromide and penthienate methobromide.

Mass spectrometry

Mass spectra of GLC resolved compounds were recorded using a Pye 104 gas chromatograph linked via a silicone rubber membrane separator to an MS 12 mass spectrometer. The ion source was operated at a temperature of 220°, a filament current of 300 μA, and an electron beam energy of 24 eV. Helium was used as a carrier gas.

General procedure for the assay of neostigmine in human plasma

A sample of plasma (usually 3 ml) in a 10-ml glass centrifuge tube was made alkaline (pH 10–12) with 20 μl 5 *N* sodium hydroxide solution and the marker solution (60 μl; equivalent to 1.5 μg base of pyridostigmine bromide in water) was added. The alkaline solution was extracted with diethyl ether (2 × 6 ml) by shaking vigorously by hand for 2 min, centrifuged at 3000 *g* for 5 min to break the emulsion, and the ethereal extracts were discarded. The remaining traces of ether were removed by purging nitrogen gas over the aqueous phase. Potassium iodide–glycine buffer (1 ml) was added to the ether-washed plasma and the resultant iodide–glycine drug complexes were extracted into 6 ml dichloromethane.

After centrifugation, the plasma (upper layer) was discarded and the dichloromethane extract was dried by shaking with a quantity of anhydrous sodium sulphate (*ca.* 2 g). The water-free extract was transferred carefully into an evaporating tube and evaporated to dryness by a gentle stream of nitrogen gas. Methanol (20 μl) was added to re-dissolve the dried extract by vortexing (5 sec), followed by centrifugation. A sample (2–5 μl) of the methanolic solution was injected onto the gas chromatographic column with a 10-μl S.G.E. syringe. The concentration of neostigmine present in a sample was determined from the ratio of the integrated peak area of neostigmine to the internal marker.

Procedure for the assay of pyridostigmine in human plasma

A procedure similar to the one described above was carried out for the determination of pyridostigmine in human plasma, using neostigmine bromide as the internal marker.

Calibration graphs

Standard solutions of neostigmine bromide and pyridostigmine bromide were prepared by dissolving the salts in distilled water. These were diluted to give a series of solutions in plasma (3 ml) covering the concentration range 50–1000 ng/ml. The solutions were then analysed as described in the general procedure, and the peak area ratios were plotted against the corresponding concentrations.

Recovery, selectivity, reproducibility and storage

Recovery. Methanolic solutions of neostigmine bromide and pyridostigmine bromide (concentration range 50–1000 ng/ml) were prepared, concentrated and assayed as described in the general procedure. Samples of plasma containing a similar range of concentrations of the drugs were assayed and the results were related to the 100% values obtained from the standard methanolic solutions. Recovery studies were also carried out using [^{14}C]neostigmine iodide and [^{14}C]pyridostigmine iodide. The total radioactivity before extraction was expressed as 100%. The percentage of radioactivity recovered after extraction indicates the efficiency of the extraction procedure; after assay by GLC, the values obtained from the radioactive samples measures the total recovery of the drugs from the entire assay procedure.

Selectivity. Samples of plasma, obtained from volunteers or from time-expired blood, were analysed to determine if they produced peaks after chromatography which interfered with those of neostigmine or pyridostigmine. In some experiments, basic drugs (for instance, pethidine and amethocaine) were added to plasma samples and assayed, in order to detect if these drugs would interfere with the chromatograms of neostigmine and pyridostigmine.

Reproducibility. Replicate samples of neostigmine and pyridostigmine in plasma (50–1000 ng/ml) were assayed and the peak area ratios of the drugs to their corresponding markers were calculated. The reproducibility of the pyrolysis dequaternisation of neostigmine and pyridostigmine to their tertiary amines was investigated by concentrating six replicate samples of neostigmine bromide (100 and 1000 ng/ml) to 20 μl; the samples were then analysed by gas chromatography using dequaternised pyridostigmine base (the dimethylcarbamate ester of 3-hydroxypyridine) as the internal standard. A similar study was carried out for pyridostigmine bromide using dequaternised neostigmine base (the dimethylcarbamate ester of 3-dimethylaminophenol) as the internal marker.

Storage. Samples of freshly collected plasma or pooled plasma from the Blood Bank were analysed immediately and after storage at −20° for 24 h and 7 days. Concentrates of the extracts containing drugs were assayed immediately and after storage at −20° for 24 h and 7 days.

RESULTS AND DISCUSSION

Choice of GLC system

All three systems investigated were able to resolve the dequaternised analogues

of neostigmine and pyridostigmine (see *Mass spectrometry* for explanation). System 3 (3% OV-17) was chosen for routine analysis because System 1 (3% OV-1) and System 2 (3% SE-30) were non-polar and offered little selectivity; thus, substances in plasma (for instance, cholesterol and fatty acids) could interfere with the analytical peaks. Compounds such as edrophonium chloride, glycopyrronium bromide, isopropamide iodide, mepenzolate bromide and penthienate methobromide were considered unsuitable as internal markers because they were thermally unstable and gave rise to more than one peak in their chromatograms (edrophonium chloride, isopropamide iodide and mepenzolate bromide).

Glycopyrronium bromide gave one peak at 32 min, while penthienate methobromide was not resolved. However, a methanolic solution of neostigmine bromide, its potassium iodide–glycine complex or its dequaternised tertiary amine (the dimethylcarbamate ester of 3-dimethylaminophenol) gave only one peak at a retention time of 8.7 min; similarly, a methanolic solution of pyridostigmine, its potassium iodide–glycine complex or its dequaternised tertiary amine (the dimethylcarbamate ester of 3-hydroxypyridine) also gave only one peak at a retention time of 2.6 min. It was therefore decided to use pyridostigmine bromide as the internal marker for analyses of neostigmine in plasma, and neostigmine as the internal standard for pyridostigmine, provided the plasma sample contained only one of the drugs at one time.

The respective retention times, symmetry factors and resolution of neostigmine bromide or its potassium iodide–glycine complex (as the dimethylcarbamate ester of 3-dimethylaminophenol) and pyridostigmine bromide or its potassium iodide–glycine complex (as the dimethylcarbamate ester of 3-hydroxypyridine) are summarised in Table I and Fig. 1.

TABLE I

PERFORMANCE OF SYSTEM 3 (3% OV-17 ON DIATOMITE CQ)

Temperatures: injection, 275°; oven, 205°; detector, 275°. Gas flow-rates: nitrogen (carrier), 30 ml/min; hydrogen, 2 ml/min (nitrogen detector) and 30 ml/min (FID); air, 100 ml/min (nitrogen detector) and 300 ml/min (FID).

Compound in methanol	*Retention time (min)*	*Symmetry factor* (0.95–1.05)*	*Resolution between marker* (>1.0)*
Neostigmine bromide Neostigmine–KI complex Dimethylcarbamate ester of 3-dimethylaminophenol	8.7	1.0	3.63
Pyridostigmine bromide Pyridostigmine–KI complex Dimethylcarbamate ester of 3-hydroxypyridine	2.6	0.96	

* British Pharmacopoeia[17] specification for GLC analysis.

Gas chromatography–mass spectrometry

The GLC system (System 3) on which the six compounds (Table I) were best separated was coupled with the mass spectrometer and the mass spectrum of each compound was recorded. The mass spectrum of neostigmine bromide and the neostigmine–potassium iodide complex was identical with that obtained from the dimeth-

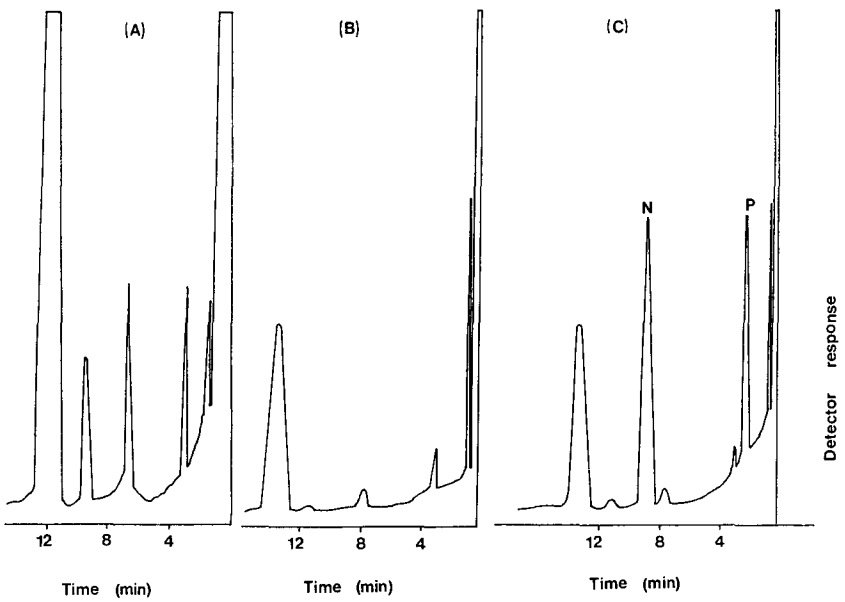

Fig. 1. Chromatograms of human plasma extracts using 3% OV-17 on Diatomite CQ. (A) Normal plasma, FID; (B) normal plasma, nitrogen detector; (C) plasma containing neostigmine (N) and pyridostigmine (P).

ylcarbamate ester of 3-dimethylaminophenol. All three spectra had a molecular ion at *m/e* 208, and a base peak at *m/e* 72 arising from fragmentation of the carbamyl group, $(CH_3)_2NCO$. The mass spectra of pyridostigmine bromide and the pyridostigmine–potassium iodide complex were identical to the mass spectrum of the dimethylcarbamate ester of 3-hydroxypyridine and the molecular ion was present at *m/e* 166. The ion at *m/e* 72 was the base peak in the mass spectra of all six compounds (Figs 2 and 3). Gas chromatography–mass spectrometry of a plasma extract containing neostigmine and pyridostigmine as potassium iodide–glycine complexes gave one

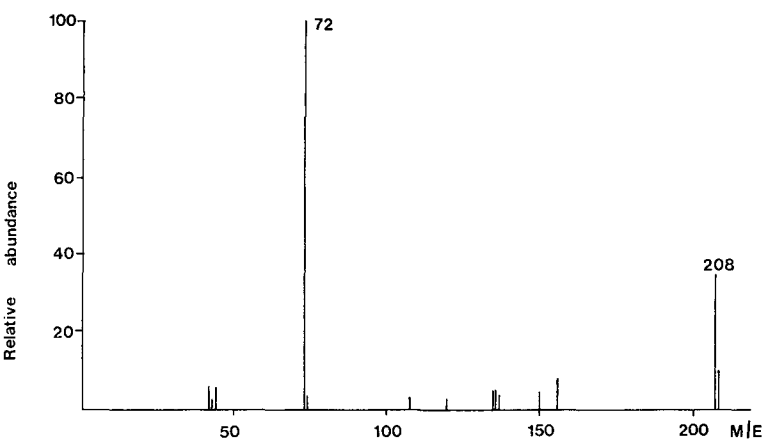

Fig. 2. GLC–MS of neostigmine bromide.

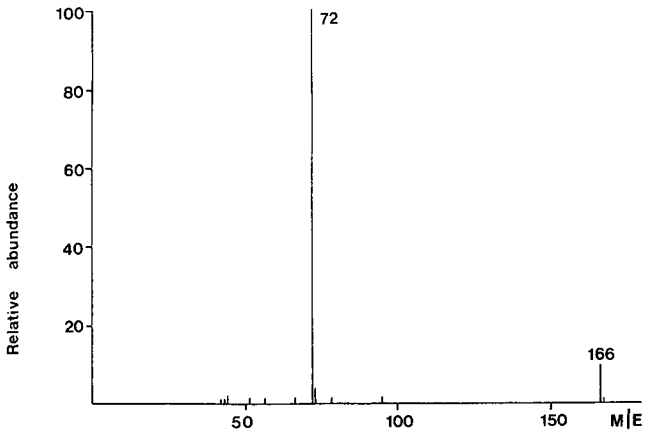

Fig. 3. GLC–MS of pyridostigmine bromide.

compound whose mass spectrum showed a molecular ion at *m/e* 166 and a second compound with a molecular ion at *m/e* 208. Both spectra had a base peak at *m/e* 72.

It was concluded that both neostigmine and pyridostigmine (either as the bromide salt or as the potassium iodide–glycine complex) were thermally dequaternised to their corresponding tertiary analogues, which were then resolved by the GLC system. Other authors have used similar methods to measure quaternary amines in plasma. In one study[5], the bioavailability of thiazinamium methylsulphate (an anticholinergic quaternary amine occasionally used in the treatment of bronchial asthma) was investigated; a similar ion-pair extraction and GLC procedure was used, although the details of the method and the chemical principle involved in N-demethylation were not discussed.

Acetylcholine and its analogues can also be measured by an assay based on pyrolysis gas chromatography, and the pyrolytic products of these compounds are their dequaternised tertiary amines[15]. The pyrolysis of quaternary ammonium halides, like the Hofmann degradation, demonstrated that the halide anion of these compounds is a sufficiently strong nucleophile at the high temperature of the injection port of the GLC system to yield a tertiary amine from the quaternary salt.

Recovery, selectivity, reproducibility and storage

Recovery. The mean relative recoveries of neostigmine and pyridostigmine from plasma were 85.7% and 88.7%, respectively (Table II). Recovery of neostigmine was lowest at 50 ng/ml (78.1%) and highest at 800 ng/ml (94.8%); the corresponding values for pyridostigmine were 82.0% (at 50 ng/ml) and 92.0% (at 400 ng/ml). Studies using radioactive neostigmine and pyridostigmine indicated that only 30% of the original radioactivity was recovered after extraction from plasma. It is possible that the loss of drug complexes during transfer procedures may account for the low recovery; alternatively, colour or chemical quenching may be produced by the test samples. Using a similar procedure, the recovery of tubocurarine from tissue extracts was approx. 89%[13]. In the present experiments, when the radioactive extracts were analysed by the GLC system, 95% recovery was achieved at a concentration of 1.6 nmole/ml of pyridostigmine.

TABLE II

RECOVERY STUDIES

Concentration (ng/ml)	*Relative recovery of neostigmine (%)*	*Relative recovery of pyridostigmine (%)*
50	78.1	82.0
100	78.6	91.6
200	81.6	84.7
400	90.2	92.0
800	94.8	90.0
1000	91.1	91.8

Selectivity. The preliminary ether wash eliminated commonly used basic drugs which might be extracted simultaneously from plasma and be analysed by the GLC system. Chan *et al.*[16] showed that this GLC system (OV-17, 3%) could resolve basic drugs (for instance, pethidine) that are removed from plasma by washing with ether. In the present experiments, there were no chromatographic peaks from a normal plasma extract which interfered with the measurement of peaks corresponding to neostigmine and pyridostigmine.

Reproducibility. When plasma samples containing neostigmine (using pyridostigmine as the internal marker) were assayed on six different occasions, the reproducibility of the peak area ratio was 100 ± 9% at 50 ng/ml and 100 ± 7% at 1000 ng/ml. The corresponding values for pyridostigmine were 100 ± 10% (50 ng/ml) and 100 ± 6% (1000 ng/ml). The reproducibility of pyrolysis at the injection port, where a glass liner was inserted, indicated that both the bromide and the potassium iodide complexes of neostigmine and pyridostigmine yielded tertiary amines, which gave linear peak area ratios with their corresponding internal markers over the range 50–1000 ng/ml (Figs. 4 and 5). These graphs were found to be reproducible when repeated six times during the studies.

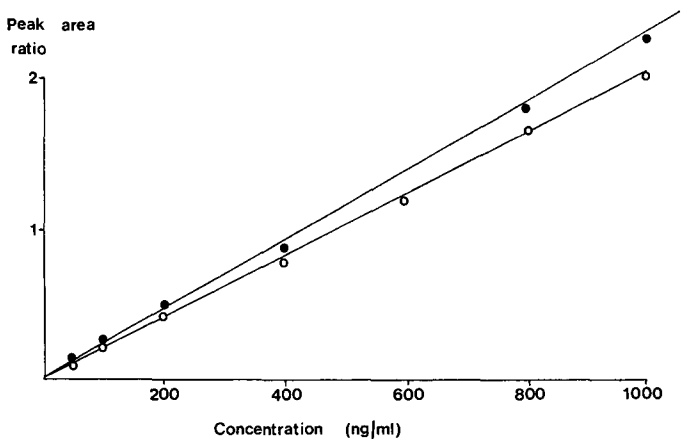

Fig. 4. Calibration graphs of neostigmine using pyridostigmine as the internal marker. ●, Methanolic solutions of the bromides of neostigmine and pyridostigmine; ○, plasma extracts of potassium iodide–glycine complexes of neostigmine and pyridostigmine. Each point represents the mean of six experiments.

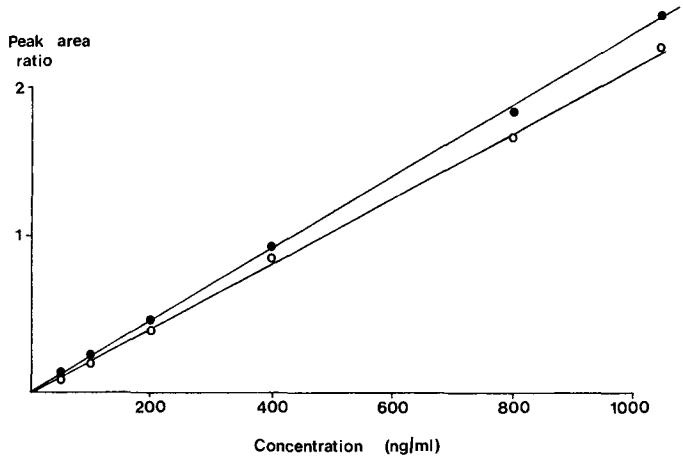

Fig. 5. Calibration graphs of pyridostigmine using neostigmine as the internal marker. ●, Methanolic solutions of the bromides of neostigmine and pyridostigmine; ○, plasma extracts of potassium iodide–glycine complexes of neostigmine and pyridostigmine. Each point represents the mean of six experiments.

Storage. Samples of plasma from healthy volunteers or from the Blood Bank (either freshly separated, or stored at −20° for 24 h or 7 days) did not interfere with the measurement of analytical peaks corresponding to the dequaternised neostigmine

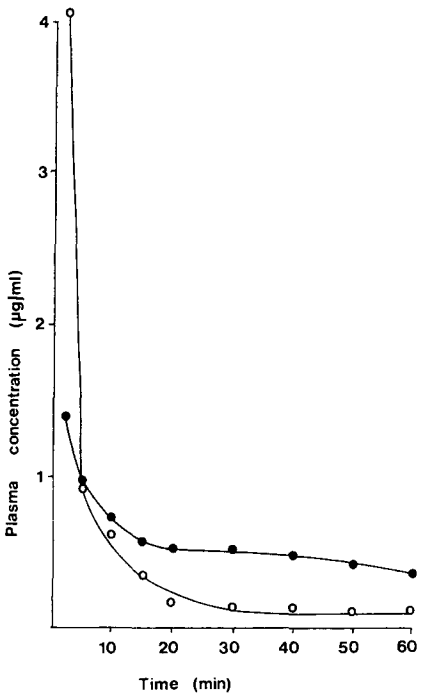

Fig. 6. Plasma concentration of neostigmine and pyridostigmine after intravenous administration of the quaternary amines. ○, Neostigmine bromide (5 mg); ●, pyridostigmine bromide (20 mg).

or pyridostigmine (Fig. 1). There was no increase or decrease in peak area ratios of drug to internal standard in extracts stored at −20° for 7 days.

Application

The procedure has been used to measure the concentration of both neostigmine and pyridostigmine in human plasma after a single intravenous injection of the quaternary amine. In both studies, tubocurarine was used to produce neuromuscular block in anaesthetised patients, and the anticholinesterase drugs were used to reverse the effects of the muscle relaxant on voluntary muscle function.

After intravenous injection of neostigmine bromide (5 mg) or pyridostigmine bromide (20 mg), samples of blood were removed at 2, 5, 10, 15, 20, 30, 40, 50 and 60 min; plasma was separated as soon as possible by centrifugation. In the one patient studied, the concentration of neostigmine in plasma fell rapidly from 4.47 μg/ml at 2 min to 0.61 μg/ml at 10 min. Subsequently, the concentration decreased to 0.11 μg/ml 60 min after intravenous administration of the drug. After intravenous pyridostigmine, the initial concentration of the quaternary amine in plasma was lower (1.39 μg/ml at 2 min); the concentration then declined relatively slowly (Fig. 6). It should be emphasised that these results are only based on the results of a single experiment, and a more detailed study concerned with the removal of both drugs from plasma is in progress.

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