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QUANTITATIVE CAPILLARY COLUMN GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF GLYCOPYRRONIUM IN HUMAN PLASMA

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SUMMARY

A new sensitive and selective capillary column gas chromatographic method for the anticholinergic agent glycopyrronium bromide in human plasma is described. The procedure involves preliminary ion-pair extraction of the drug into dichloromethane, followed by concentration and analysis of the ion-pair complex by capillary column gas chromatography using a nitrogen-sensitive detector. The method depends on the thermal dequaternisation of the quaternary ammonium compound and can be used to detect 5 ng/ml in a 3-ml plasma sample. The assay procedure has been applied to the determination of the plasma concentration of glycopyrronium after intravenous administration to an anaesthetised patient.

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INTRODUCTION

Glycopyrronium bromide (glycopyrrolate; Robinul) is a quaternary amine with antimuscarinic properties, which may be used as an alternative to atropine, during the antagonism of non-depolarising neuromuscular blockade. In these conditions, it may have several important advantages. It does not enter the central nervous system to a significant extent, or produce central effects [1, 2]; it does not cause tachycardia or cardiac arrhythmias [3, 4]; and its duration of action is 4—7 h [5]. Nevertheless, little is known of the clearance, distribution or metabolism of the drug in man.

This paper presents a sensitive and selective analytical method for the determination of glycopyrronium in human plasma based on capillary column gas chromatography (GC) with nitrogen-sensitive detection. The procedure used is a modification of the method developed to measure the concentration of neostigmine and pyridostigmine in plasma [6] and is based on ion-pair extraction of the drug.

EXPERIMENTAL

Materials

The following materials were used: dichloromethane, diethyl ether and methanol, all of AnalaR grade and freshly redistilled; AnalaR anhydrous sodium sulphate; 0.1 mol/l and 5 mol/l sodium hydroxide solutions; 3 mol/l hydrochloric acid solution; 0.1 mol/l glycine buffer (AnalaR glycine, 7.505 g; AnalaR sodium chloride, 5.185 g and distilled water to 1 l); potassium iodide—glycine buffer (AnalaR potassium iodide, 12.8 g; 0.1 mol/l sodium hydroxide solution, 4 ml and 0.1 mol/l glycine buffer, 6 ml); potassium triiodide solution; iodine—potassium iodide—water (1:2:20, w/w/v); glycopyrronium bromide (A.H. Robins, Horsham, U.K.); [^{14}C]glycopyrronium bromide (A.H. Robins, Richmond, VA, U.S.A.); mepenzolate bromide (M.C.P. Pharmaceuticals, Wembley, U.K.); neostigmine bromide and pyridostigmine bromide (Roche, Welwyn Garden City, U.K.).

Apparatus

The following apparatus was used: a Sigma 3 gas chromatograph fitted with a phosphorus—nitrogen detector and linked to a Hitachi chart recorder Model 56 (Perkin-Elmer, Beaconsfield, U.K.). This system was operated with quartz capillary columns (25 m × 0.21 mm I.D.) and with coiled glass columns (1.5 m × 4 mm O.D.) silanized with hexamethyldisilazane (HMDS, Chromatography Services, Merseyside, U.K.) before use. These columns were packed with various stationary phases. The operating temperature for the injector and detector was 320°C and a suitable one for the column. Gas flow-rates were helium (carrier gas) 1 ml/min for capillary columns and 30 ml/min for glass columns, hydrogen 4 ml/min and air 100 ml/min. The setting of the rubidium bead in the phosphorus—nitrogen detector was adjusted to optimum condition before use. Other apparatuses used were: 15-ml capacity centrifuge tubes with well fitting screw caps (Sovirel, Levallois-Perret, France); 15-ml capacity stoppered evaporation tubes with finely tapered bases. All glassware was

cleaned by soaking overnight in a 2% solution of RBS 25 [Chemical Concentrates (RBS), London, U.K.] in water, then rinsing thoroughly with hot tap water followed by methanol and distilled water. These tubes were subsequently silanized by rinsing with a 3% (v/v) solution of HMDS in redistilled chloroform, and dried at 250°C overnight. This treatment of glassware was found to be necessary to eliminate possible loss of drug due to adsorption on the glass wall [7].

Gas chromatography

Methanolic solutions of glycopyrronium bromide and possible internal standards were injected on to the following chromatographic systems at various oven temperatures. System 1: Chromosorb W-AW (100—120 mesh) packed with 3% OV-17, in a coiled glass column (1 m × 4 mm O.D.). System 2: fused-silica capillary column coated with OV-101 (25 m × 0.21 mm I.D.). System 3: fused-silica capillary column coated with OV-1 (25 m × 0.21 mm I.D.).

All columns were conditioned at 20°C below that of the maximum recommended temperature of the relevant stationary phase for 24 h. Retention times, resolution and symmetry factors of the chromatographic peaks for these systems were then established. Compounds which were investigated as possible internal standards were: mepenzolate bromide, neostigmine bromide and pyridostigmine bromide.

Gas chromatography—mass spectrometry

Mass spectra of the GC resolved compounds were acquired using a Finnigan 1020 Series automated gas chromatograph—mass spectrometer operated in the electron-impact mode of ionisation. An electron energy of 24 eV and an ion source temperature of 80°C were used. Helium was used as carrier gas at a flow-rate of 1 ml/min.

General procedure for the determination of glycopyrronium in plasma

Blood samples were obtained by venous puncture after intravenous administration and collected in heparinised polythene tubes. The red blood cells were separated from the plasma by centrifugation (2500 *g* for 10 min). A sample of plasma (3.0 ml) in a 15-ml glass centrifuge tube was made alkaline (pH 10–12) with 20 μl of 5 mol/l sodium hydroxide solution and the internal standard, mepenzolate bromide (60 μl of a standard solution equivalent to a concentration of 2 μg/ml in water), was added. The alkaline solution was extracted with diethyl ether (10 ml) using an automatic shaker at a speed of 40 rpm for 10 min. The organic layer was then separated from the aqueous layer by centrifugation (2500 *g* for 10 min) and the ethereal extract was discarded. The remaining traces of diethyl 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 dichloromethane (10 ml).

After mixing and centrifugation at 3500 *g* for 15 min, the plasma (upper) layer was discarded and the dichloromethane extract was dried by shaking with a quantity of anhydrous sodium sulphate (approximately 2 g). The water-free

extract was transferred carefully into an evaporation tube and evaporated to dryness by a gentle stream of nitrogen gas. The stoppered evaporation tube was then stored at −20°C before GC analysis. The residue was reconstituted in redistilled methanol (30 μl), by vortexing for 5 sec, immediately before GC analysis. An aliquot (2—5 μl) of the final concentrate was injected on to the GC system. The concentration of glycopyrronium present in the plasma sample was determined from the ratio of the peak height of glycopyrronium to that of the internal standard.

Calibration graphs

A standard solution of glycopyrronium bromide was prepared by dissolving the salt in distilled water. This was diluted to give a series of solutions in plasma (3.0 ml) covering the concentration range 5—100 ng/ml. The solutions were then analysed as described in the general procedure and the peak height ratios were plotted against the corresponding concentrations.

Recovery

The recovery of [^{14}C]glycopyrronium bromide from plasma was investigated using potassium iodide- glycine buffer at pH 10 and also using potassium triiodide as counter ions at pH 5—6. The recoveries at three different drug concentrations were studied (20, 50, and 100 ng/ml).

Samples of fresh plasma (3.0 ml) were spiked with [^{14}C]glycopyrronium bromide (specific activity = 11.7 μCi/mg) in water to give drug concentrations of 20, 50 and 100 ng/ml. The total radioactivity before extraction was expressed as 100%. The percentage of radioactivity recovered after extraction indicated the efficiency of the extraction procedure.

Each plasma sample was assayed as described in the general procedure. Where potassium triiodide, instead of potassium iodide, was used as counter ion, the ether-washed plasma was acidified with 100 μl of 3 mol/l hydrochloric acid solution (pH 5) and 100 μl potassium triiodide solution were added. The resultant drug complexes were extracted into 10 ml dichloromethane as in the general procedure. In all samples the water-free dichloromethane extracts were transferred into plastic scintillation vial inserts and evaporated to dryness under nitrogen gas. Redistilled methanol (200 μl) was added followed by 7 ml scintillation fluid (Triton-X-100/toluene cocktail). The radioactivity in each vial was counted for 100 min and the number of counts recorded (cpm) and converted to dpm.

The radioactivity before extraction (i.e. 100%) was determined by making up concentrations equivalent to 20, 50 and 100 ng/ml [^{14}C]glycopyrronium in 200 μl methanol in vial inserts and adding 7 ml scintillation fluid and counting radioactivity as above.

Selectivity

Samples of plasma obtained from volunteers were analysed to determine if they produced peaks after chromatography which interfered with that of glycopyrronium. In some experiments, basic drugs (e.g. pethidine and lignocaine) were added to plasma samples and assayed in order to detect if these drugs interfered with the chromatogram of glycopyrronium.

Reproducibility

Eight replicate samples of glycopyrronium (40 ng/ml) in plasma were assayed as in the general procedure and the peak height ratio of the drug to internal standard (mepenzolate) was calculated for each sample.

Storage

Samples of freshly collected plasma were analysed immediately and after storage at −20°C for 24 h and 7 days. Concentrates of the extracts containing drugs were assayed immediately and after storage at −20°C for 24 h and 7 days.

RESULTS AND DISCUSSION

Choice of GC system

All three systems investigated resolved the dequaternised analogue of glycopyrronium (see Mass spectrometry for explanation). System 3 (OV-1 capillary column), however, offered little selectivity and substances in plasma such as cholesterol and fatty acids interfered with the drug peak. Systems 1 (3% OV-17 glass column) and 2 (OV-101 capillary column) were both suitable for the determination of glycopyrronium. System 2, using splitless injection but with the split vent opened after 30 sec, gave superior peak shape and much less interference from the components present in the solvent front. System 2 was therefore used for routine analysis. With the column at 240°C, a vent time of 30 sec and a split ratio of 50:1, a methanolic solution of glycopyrronium bromide and a methanolic solution of its potassium iodide—glycine complex following extraction both gave one peak at a retention time of 4.8 min.

Mepenzolate bromide was the most suitable compound for use as an internal standard. A methanolic solution of mepenzolate bromide and of its potassium iodide—glycine complex gave only one peak at a retention time of 7.0 min.

TABLE I

PERFORMANCE OF SYSTEM 2 (OV-101 QUARTZ CAPILLARY COLUMN)

Temperatures: injection, 320°C; oven, 240°C; detector, 320°C. Gas flow-rates: helium (carrier), 1 ml/min, split ratio 50:1 and vent time 30 sec; hydrogen 4 ml/min and air 100 ml/min; bead setting 400.

Compound	Retention time (min)	Symmetry factor* (0.95—1.05)	Resolution between marker* (≥ 1.0)
Glycopyrronium bromide	4.8	1.0	2.75
Glycopyrronium— potassium iodide complex			
Mepenzolate bromide	7.0	0.96	
Mepenzolate—potassium iodide complex			

*British Pharmacopoeia specification for gas—liquid chromatographic analysis [8].

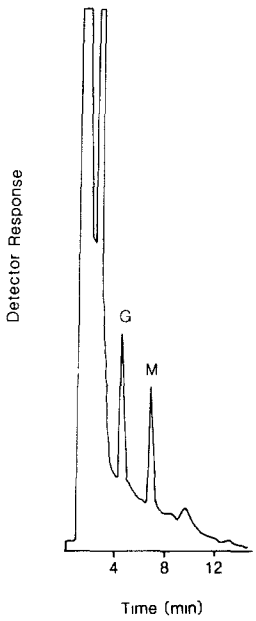

Fig. 1. Chromatogram of glycopyrronium (G) and the internal standard mepenzolate (M) after ion-pair extraction from human plasma.

Both neostigmine and pyridostigmine bromide produced peaks at less than 3 min and could not be separated from other components in the solvent front.

The respective retention times, symmetry factors and resolution of glycopyrronium bromide and its potassium iodide—glycine complex and of mepenzolate bromide and its potassium iodide—glycine complex are summarised in Table I and Fig. 1.

Gas chromatography—mass spectrometry

It was demonstrated that both glycopyrronium and mepenzolate (either as the bromide salt or as the potassium iodide—glycine complex) were thermally

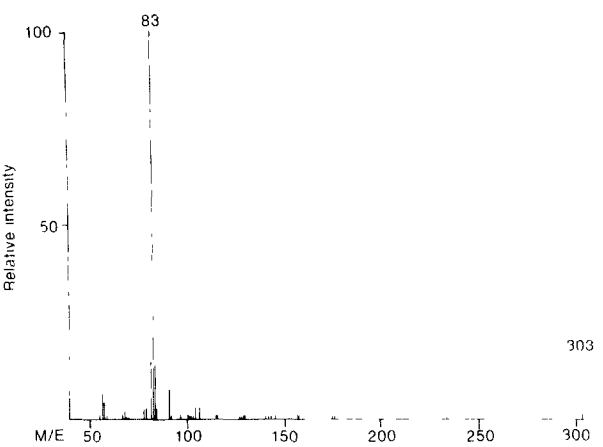

Fig 2 Electron-impact mass spectrum of glycopyrronium bromide.

dequaternised to their corresponding tertiary analogues which were then resolved by the GC system, by coupling System 2 with a mass spectrometer and recording the mass spectrum of each compound. The mass spectra of glycopyrronium bromide and of its potassium iodide—glycine complex following ion-pair extraction were found to be identical. Both spectra had a molecular ion at *m/e* 303, and a base peak at *m/e* 83 resulting from breakdown of the ester grouping and subsequent release of an N-methylpyrrolidinyl fragment (Figs. 2 and 3). The mass spectra of mepenzolate bromide and of its ion pair complex were identical, both having a molecular ion at *m/e* 325 and a base peak at *m/e* 97, resulting from the breakdown of the ester grouping and subsequent release of the N-methylpiperidinyl fragment (Figs. 3 and 4).

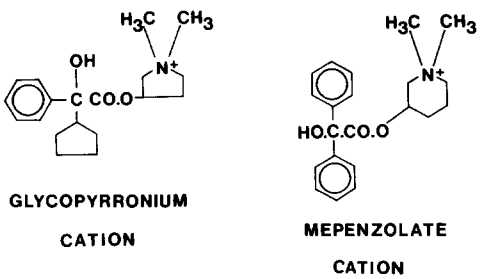

Fig. 3. Structure of glycopyrronium and mepenzolate.

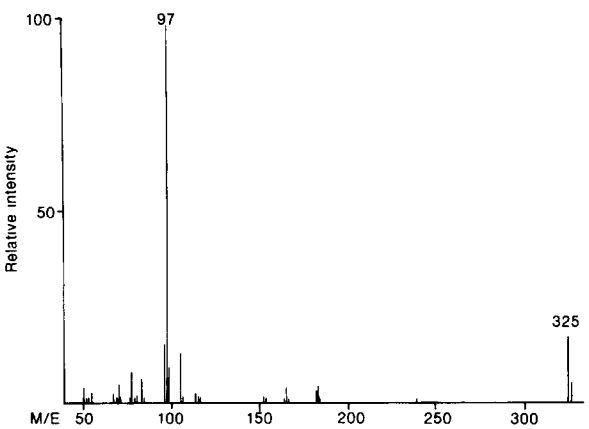

Fig. 4. Electron-impact mass spectrum of mepenzolate bromide (internal standard).

TABLE II

RECOVERY STUDIES

Each value is mean ± S.E.M. of four experiments.

Concentration (ng/ml)	Percent recovery	
	Potassium iodide—glycine buffer	Potassium triiodide
20	52.29 ± 1.06	48.59 ± 1.73
50	51 41 ± 1.61	47.46 ± 2.27
100	49.62 ± 1.46	47.99 ± 1.14

Recovery

The recovery of [^{14}C] glycopyrronium from plasma using potassium iodide—glycine buffer ranged from 49.6 to 52.3% at concentrations between 20 and 100 ng/ml (Table II). Using potassium triiodide as pairing ion, the recovery of [^{14}C] glycopyrronium ranged from 47.5 to 48.6% (Table II). Therefore as no substantial difference was found in drug recovery using a stronger pairing ion, potassium iodide—glycine buffer was used in the general procedure.

Selectivity

The preliminary ether wash eliminated commonly used basic drugs (for instance pethidine and lignocaine) which might be extracted simultaneously from plasma and be analyzed by the GC system. In the present procedure, there were no chromatographic peaks from a normal plasma extract which interfered with the measurement of peaks corresponding to glycopyrronium and mepenzolate.

Reproducibility

When eight replicate samples of glycopyrronium (40 ng/ml) in plasma were assayed using mepenzolate (2 μg/ml) as internal standard, the reproducibility of the peak height ratio was 100 ± 9%. The calibration graphs of glycopyrronium using mepenzolate as internal standard were found to be reproducible when repeated five times during the studies. There was invariably a linear relationship between the concentration of glycopyrronium in plasma (in the range from 5—100 ng/ml) and the peak height ratio of glycopyrronium: mepenzolate.

Storage

There was no difference in peak height ratios to internal standard in extracts assayed immediately and after storage at —20° C for 24 h and 7 days.

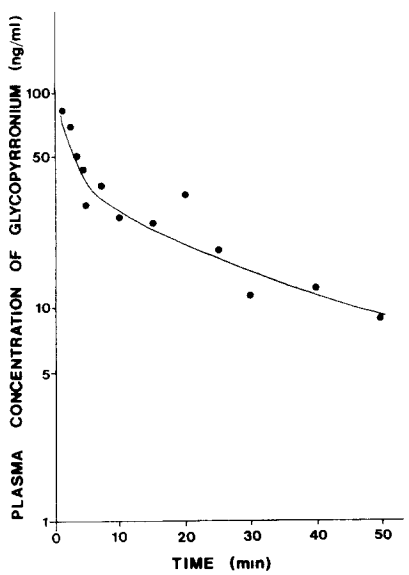

Fig. 5 Semi-logarithmic plot of the plasma concentration of glycopyrronium after intravenous administration to an anaesthetised patient. Dose of glycopyrronium = 0.3 mg.

Application

The procedure has been used to measure the concentration of glycopyrronium in human plasma after intravenous injection of the quaternary amine. Glycopyrronium was used in anaesthetised patients to prevent undesirable muscarinic effects during the antagonism of non-depolarising neuromuscular blockade.

After intravenous injection of glycopyrronium bromide (0.3 mg), samples of blood were collected at 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 40 and 50 min; plasma was separated as soon as possible by centrifugation. In the one patient studied, the concentration of glycopyrronium fell rapidly from 80.8 ng/ml at 1 min to 36.5 ng/ml at 7 min. Subsequently the concentration of the drug declined more slowly to 8.6 ng/ml after 50 min (Fig. 5). It should be emphasised that these results are only based on a single study. A more detailed investigation of the plasma concentration of glycopyrronium after intravenous administration is in progress.

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