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DETERMINATION OF ORG NC 45 (A MYONEURAL BLOCKING AGENT) IN HUMAN PLASMA USING HIGH-PERFORMANCE NORMAL-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The assay for the quantification of Org NC 45 in human plasma is described in detail. It comprises ion-pair extraction and normal-phase liquid chromatography in conjunction with UV detection. An analogue, 17β-deacetyl-Org NC 45, is used as standard in the assay procedure. The accuracy and precision of the assay at 400 ng of Org NC 45 per ml of plasma are 0.7% and 2.8%, respectively. The detection limit is approx. 50 ng Org NC 45 per ml of plasma. The assay can be used for the pharmacokinetic evaluation of Org NC 45 in man.

INTRODUCTION

Org NC 45 (1-[(2β,3α,5α,16β,17β)-3,17-bis(acetyloxy)-2-(1-piperidinyl)-androstan-16-yl]-1-methylpiperidinium bromide) is a non-depolarizing neuromuscular blocking agent of the pancuronium type with a wide margin of cardiovascular safety [1—4]. Clinical data indicate a faster onset, shorter duration and more rapid offset of action than with equivalent doses of pancuronium (Pavulon®). For the latter compound a fluorimetric assay method [5] has been described where the sum of pancuronium and its biotransformation products is determined in human plasma non-selectively. Thin-layer chromatography (TLC) allowed complete separation of pancuronium from its biotransformation products [6]; however, quantification by TLC is semi-quantitative. A similar batch procedure [7] was developed for the quantification of Org NC 45 in plasma. Direct extraction from plasma with the fluorescent dye rose bengal was not feasible; therefore, a sequence of extractions was used. The latter procedure does not separate Org NC 45 from its theoretically probable biotransformation products (Fig. 1) and it is subject to large variations.

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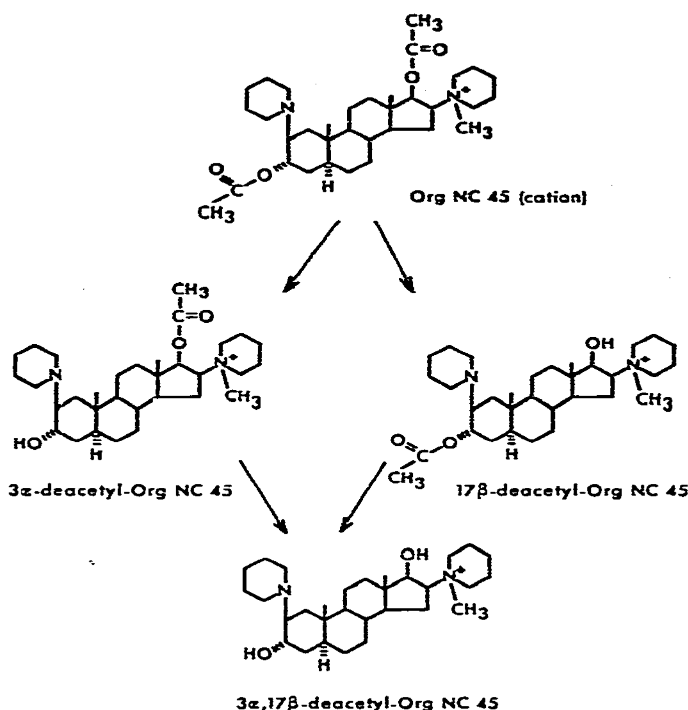

Fig. 1. Structural formulae of Org NC 45 and its theoretically probable biotransformation products.

A specific and sensitive assay method is required for the assessment of the pharmacokinetic parameters of Org NC 45 in man. De Zeeuw et al. [8] and Greving et al. [9] advocated the use of normal-phase ion-pair liquid adsorption chromatography for the determination of quaternary ammonium compounds. This report describes the assay for selective quantification of nanogram amounts of Org NC 45 in human plasma using ion-pair extraction and normal-phase liquid chromatography.

MATERIALS AND METHODS

Chemicals and glassware

Org NC 45 and its deacetylated products were obtained from Organon (Newhouse, Great Britain). Methanol and 1,2-dichloroethane (DCE) were of crude quality [Diosynth (Oss, The Netherlands) quality grade B] and distilled prior to use. All other chemicals were of analytical grade quality and purchased from Merck (Darmstadt, G.F.R.). The Glycine stock solution contained 750.5 mg of Gly and 585 mg of NaCl per 100 ml of water. The KI—Gly buffer solution was prepared freshly each day of analysis by mixing 4 ml of 0.1 *N* NaOH with 6 ml of Gly stock solution and 6,4 g of KI. All glassware was cleaned prior to use with a detergent (Alconox) by immersion in an ultrasonic tank and washing thoroughly with dilute hydrochloric acid and distilled water.

Acidification and storage of plasma samples

Sodium citrate was used as the anti-coagulant. After blood sampling in a clinical study, plasma samples were adjusted immediately to approx. pH 6 by addition of approx. 150 μl of 1 M NaH_2PO_4 per ml of plasma. This prevented the hydrolysis of Org NC 45 (Fig. 1). Plasma samples were stored at $-20°C$ until required for use.

Sample processing

The assay standard, 17β-deacetyl-Org NC 45 (dissolved in 0.01 N HCl) was added to the mixture of 2 ml of acidified (pH 6) human plasma, 1 ml of KI—Gly solution and 7 ml of DCE in a 30-ml glass-stoppered centrifuge tube. Mild extraction was attained by gently rotating the tubes on a rotary disc for 30 min. After centrifugation for 10 min at 1300 g, the upper (aqueous) layer was carefully aspirated. The organic layer was then removed using a 10-ml syringe. The organic phase was concentrated by solvent evaporation under a gentle stream of nitrogen (waterbath, 40°C). The residue was dissolved in 70 μl of methanol by ultrasonification for 1 min and injected into the high-performance liquid chromatographic (HPLC) system.

HPLC apparatus and phase system

The HPLC set-up consisted of a Waters Model 6000A pump, a Waters U6K universal injector and a Pye Unicam LC 3-UV variable-wavelength detector (detection at 215 nm). All chromatograms were obtained using a Kipp BD8 multi-range recorder. A prepacked LiChrosorb Si-60 (particle size 7 μm) column (Chrompack, Middelburg, The Netherlands) of 25 cm × 4.6 mm I.D. was used. The mobile phase consisted of methanol, 1% of a 25% aqueous ammonia solution in water and 60 mM ammonium chloride and was prepared freshly each day of analysis. The eluent was ultrasonified and filtered through a Millipore filter (0.2 μm) prior to use. The eluent flow-rate was 2 ml/min. All experiments were performed at ambient temperature.

Calibration and quantification

For calibration purposes, 2 ml of human plasma free of drug (blank) was processed, spiked with 1 μg of Org NC 45 (0.01 N HCl) and various amounts of standard in a ratio of Org NC 45 to the standard ranging from 2.5 to 0.33. Peak heights of Org NC 45 and the standard were measured. The Org NC 45 plasma concentration was calculated using a calibration curve based on a BASIC programme with DEC PDP-11 RSTS computer facilities.

RESULTS

Selectivity

For pharmacokinetic evaluation Org NC 45 should be separated from its probable biotransformation products and endogenous plasma components. The chromatogram showing the separation of Org NC 45 from its theoretically probable biotransformation products is shown in Fig. 2. Fig. 3 shows characteristic chromatograms of blank human plasma, spiked human plasma and a plas-

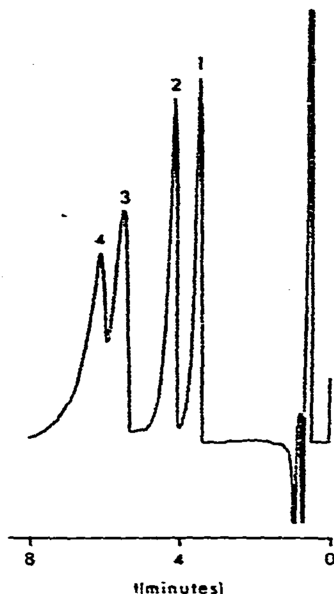

Fig. 2. Separation of Org NC 45 from its probable hydrolysis products. Stationary phase, LiChrosorb Si-60 (7 μm); mobile phase; methanol, 1% of a 25% aqueous ammonia solution and 60 m*M* ammonium chloride; ambient temperature; flow-rate, 2 ml/min. Peaks: 1 = 17β-deacetyl-Org NC 45; 2 = Org NC 45; 3 = 3α, 17β-deacetyl-Org NC 45; 4 = 3α-deacetyl-Org NC 45.

ma sample derived from a clinical study. It can be seen that the assay method offers ample selectivity for the determination of Org NC 45.

Retention and column performance

Retention of standard and Org NC 45 was subject to slight changes in the course of routine application. In addition, retention varied slightly from column to column. In principle, retention can be enhanced either by decreasing the ammonium chloride concentration or by adding more of the ammonia solution. For adjusting the retention time, a change in the ammonium chloride concentration proved to be the more appropriate method. A twofold increase in the ammonium chloride concentration will result in approximately a twofold decrease in retention time. At a flow-rate of 2 ml/min, the retention time for Org NC 45 should not exceed 10 min, since peak tailing will result in a considerable loss in accuracy and precision when measuring peak heights.

A gradual decrease in column performance was observed during routine application. Washing the column with 50 ml of methanol, 50 ml of acetone, 50 ml of hexane and then the same solvents in the reverse order, and re-equilibrating the column with the eluent, partly restored the performance. Renewing the top of the column packing by manual refilling was also beneficial. When not in use, the HPLC system was flushed thoroughly with methanol. Overnight, the eluent was recirculated at a solvent flow-rate of 0.5 ml/min.

Assay linearity, accuracy and precision

The calibration curve was fitted using polynomial regression analysis accord-

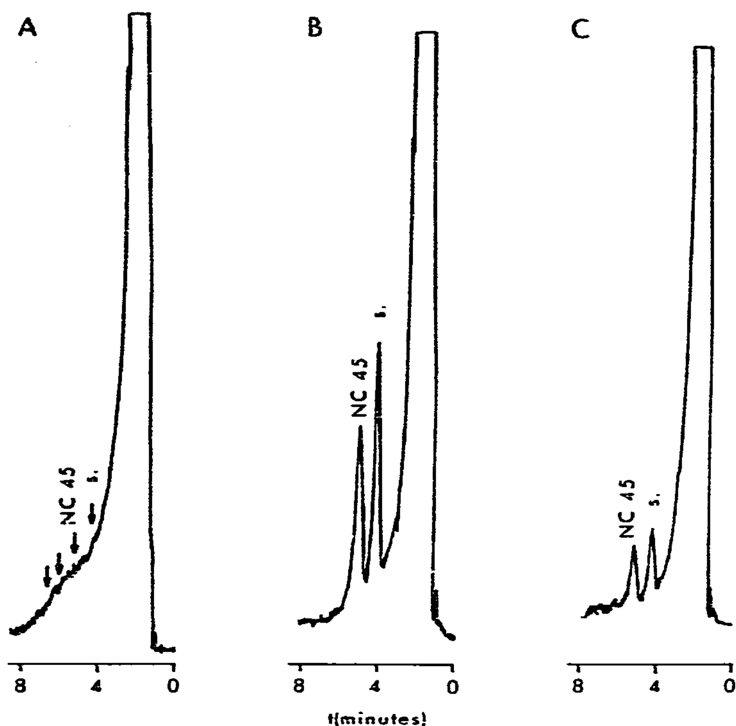

Fig. 3. Chromatograms of blank human plasma, spiked human plasma and human plasma sample from a clinical study. Stationary phase, LiChrosorb Si-60 (7 μm); mobile phase, methanol, 1% of a 25% aqueous ammonia solution and 60 m*M* ammonium chloride; ambient temperature; flow-rate, 2 ml/min. (A) Blank human plasma; the expected positions of the standard (s.), Org NC 45 and other probable biotransformation products (see Fig. 2) are indicated by arrows. (B) Human plasma, spiked with 1 μg of Org NC 45 and 1 μg of standard (s.) per 2 ml of plasma. (C) Clinical plasma sample; 3 ml of plasma were processed after spiking with 300 ng of standard (s.). The Org NC 45 concentration was calculated to be 145 ng per ml of plasma.

ing to $y = ax^2 + bx + c$, where typically $a = 1.88 \times 10^{-2}$, $b = 0.78$, and $c = -3.86 \times 10^{-2}$. The deviation from linearity is small. The peak height ratios proved to be independent of the amount of Org NC 45 at different ratios of Org NC 45 and standard. The assay accuracy [= relative difference (%) between actual and observed mean values] and precision [= relative (%) standard deviation] were determined by ninefold processing of 800 ng of Org NC 45, added to 2 ml of blank human plasma and spiked with 800 ng of standard. The within-day assay accuracy was 0.7% and the precision 2.8%.

Detection limit

The detection limit was arbitrarily defined as equivalent to three times the peak-to-peak noise level. In routine analysis 2 ml of plasma were processed. The detection limit was approx. 200 ng of Org NC 45 per 2 ml of plasma. Since 4 ml can be processed with adapted amounts of extractants without influencing the noise level, the detection limit ultimately amounted to approx. 50 ng of Org NC 45 per ml of plasma.

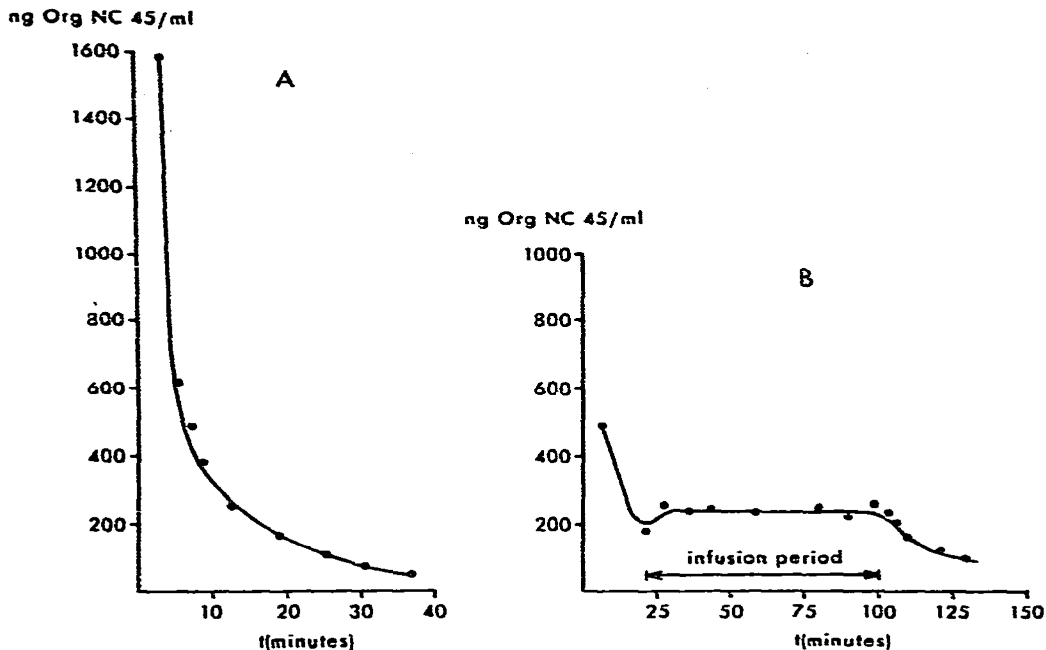

Fig. 4. Profiles of Org NC 45 plasma concentration versus time. (A) Intravenous bolus dose (0.11 mg/kg). (B) Intravenous bolus dose (0.11 mg/kg); intravenous infusion rate = 1.51 μg/kg per min. Infusion period was as indicated.

Quality control

Concurrently, on each day of analysis, human plasma samples (free of drug) spiked with a known amount of Org NC 45 at different levels were processed and quantified. When there were significant differences between the actual and true value for the Org NC 45 concentration, all samples (clinical, calibration, test) were reprocessed. This continuous quality control of the assay guarantees the acquirement of accurate data, also around the detection limit.

Routine application

The pharmacokinetics and pharmacodynamics of Org NC 45 have been determined with patients undergoing elective facial or oral surgery by concurrent plasma level determination of Org NC 45 and monitoring the twitch response of the adductor pollicis brevis muscle as a parameter for the degree of muscular block [10]. Typical examples of the time course curve of Org NC 45 plasma levels after intravenous administration and/or intravenous infusion are shown in Fig. 4.

DISCUSSION

It should be noted that the standard, 17β-deacetyl-Org NC 45, is a probable hydrolysis and/or biotransformation product derived from Org NC 45 (Fig. 1). However, in all experiments performed either with blank human plasma samples spiked with Org NC 45 in the framework of a time—course hydrolysis ex-

periment or with human plasma samples derived from a clinical study, the presence of 17β-deacetyl-Org NC 45 could never be demonstrated. Thus, the use of 17β-deacetyl-Org NC 45 as standard for the Org NC 45 assay was justified. In the course of the assay development, addition of the standard to plasma gave rise to inaccurate and irreproducible results. Although only conjectures (drug—plasma protein binding) can be made about the explanation of this phenomenon, the problem was circumvented by the addition of the standard to the incubation mixture of plasma and extractants, just prior to extraction. It should be stressed that, in the course of the sample processing, the residue after concentration should not be dissolved in the HPLC eluent, since this will lead to erroneous results.

Org NC 45 is liable to hydrolyze under alkaline and neutral conditions (plasma, water). However, under the "alkaline" conditions for elution, no on-column hydrolysis of Org NC 45 was observed. There are indications (decrease in retention with increasing ammonium chloride concentration) that the retention mechanism is governed by ion-pair adsorption. However, the composition of the mobile phase was not changed systematically as to verify these indications quantitatively [9]. These aspects were judged to fall beyond the scope of this paper.

CONCLUSION

The assay method developed allows the selective determination of Org NC 45 in human plasma with good accuracy and precision. It has been shown to be applicable [10] for the assessment of the pharmacokinetic parameters of Org NC 45 in man.

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NOTE ADDED IN PROOF

Occasionally unacceptable noise and baseline drifts were observed even after excessive ultrasonic degassing of the eluent before chromatography. This appeared to be dependent upon the reabsorption of oxygen into the eluent during chromatography. Continuous helium degassing solved the problem. The role of dissolved gases has been discussed before [11]. Recently, results of a systematic study were presented on solvent degassing and other factors affecting LC detector stability at low UV wavelengths [12].

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