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Quantification of the neuromuscular blocking agent rocuronium and its putative metabolite 17-desacetylrocuronium in heparinized plasma by capillary gas chromatography using a nitrogen sensitive detector

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Abstract

We have developed a sensitive and specific capillary GC (cGC) assay for the quantification of the quarternary aminosteroidal compound rocuronium (roc), a neuromuscular blocking agent, and its putative metabolite 17-desacetylrocuronium (17OH-roc), using 3-desacetylvecuronium (3OH-vec) as an internal standard (I.S.). This novel method has been applied to a pharmacokinetic study with roc, monitoring sixty patients who were classified according to four different body mass index (BMI) groups. The isolation of these drugs from plasma was carried out using a dichloromethane liquid–liquid extraction after ion-pairing of the positively charged ammonium compounds with iodide. To achieve thermal stability, *tert.*-butyldimethylsilyl-ethers were formed at the 3OH- and 17OH-steroidal positions by reaction with N-methyl-N-(*tert.*-butyldimethylsilyl)-trifluoroacetamide at 70°C overnight. An automated cGC system fitted with a nitrogen sensitive detector with a specially prepared glass phase bead and a computer controlled data handling system was used to analyze and quantify the compounds, which were separated on a DB1 capillary column with helium as the carrier gas and a temperature program ranging from 120 to 300°C. The method is linear for 50–6400 ng/ml for roc and 80–6400 ng/ml for 17OH-roc. The detection limits were 10 ng/ml for roc and 50 ng/ml for 17OH-roc. The lower limit of quantification was 50 ng/ml for roc and 80 ng/ml for 17OH-roc. Intra-assay coefficients of variation (C.V.s) were 10% and 15% and the inter-assay C.V.s 8–18% and 16–21% for roc and 17OH-roc, respectively. © 1997 Elsevier Science B.V.

Keywords: Rocuronium; 17-Desacetylrocuronium

1. Introduction

The aminosteroidal compound rocuronium (roc) (Fig. 1) is the result of intensive pharmaceutical research on the development of a non-depolarising neuromuscular blocking agent that could replace succinylcholin for rapid intubation.

Roc shows similar properties to vecuronium.

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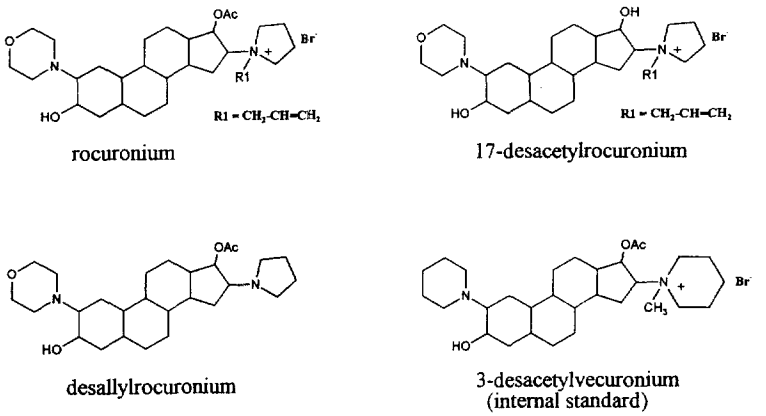

Fig. 1. Structural formulae of internal standard, rocuronium and its two putative metabolites.

Former studies [1–3] have demonstrated that the drug is characterized by a rapid onset time and good intubation conditions. At present, however, little kinetic data is available for this compound. In order to determine the time- and dose-dependent pharmacokinetics, we modified a capillary gas chromatography (cGC) assay from Furuta et al. [4], who developed this method for various neuromuscular blocking agents such as vecuronium, pancuronium, pipecuronium and their respective metabolites by using nitrogen sensitive detection. Some high-performance liquid chromatography (HPLC) applications for vecuronium or pancuronium have been described [5,6], but to date only one method has been published for roc [7], requiring a special post-column extraction with a second pump device. Furthermore, the chromatographic run is time-consuming and has a high solvent consumption.

Therefore we have developed a sensitive, specific and easy to handle assay system suitable for large-scale determinations required for the evaluation of the pharmacokinetic properties of roc in normal, obese and leptosomatic patients [8].

2. Experimental

2.1. Chemicals

Rocuronium bromide (Org 9426, purity>95%, roc), 17-desacetylrocuronium bromide (Org 9943, purity>99%, 17OH-roc), desallylrocuronium bromide (Org 20860, purity>99%) and 3-desacetylvecuronium bromide (Org 7268, purity>94%, 3OH-vec, internal standard I.S.) were supplied by Organon Teknika (Turnhout, Belgium). Acetone GR dried, acetonitrile GR dried, 1,1,1-trichloroethane, diethylketone and potassium-iodide extra pure and Extrelut cartridges were purchased from Merck (Darmstadt, Germany). Diethyl ether, ethylmethylketone (both analytical-reagent grade) and dichloromethane were obtained from Fluka (Buchs, Switzerland). N-Methyl-N-(*tert.*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was obtained from Chrompack (Frankfurt, Germany).

Demineralized ultrafiltrated water was provided by a Millipore Milli-Q UF Plus apparatus (Eschborn, Germany).

2.2. Instrumental

A Varian Star 3400 CX fitted with an autosampler 8200 CX and a nitrogen-specific detector (TSD) from Varian (Darmstadt, Germany) was used. The data were calculated with the Varian Data Conversion Software Star Chromatography Workstation 4.0. A 10 m×0.32 mm I.D. column DB1 with a film thickness of 0.25 μm from J&W Scientific (Köln, Germany) was used for the separation of the compounds. The Varian TSD bead probe was modified by applying a rubidium dotted glass bead, which was prepared by Blos Analysentechnik (Munich, Germany). The extraction was carried out with a Rotary Mixer from Breda Scientific. Dichloromethane

(CH_2Cl_2) was vacuum evaporated with a Speed Vac Plus SC 110 A from Savant (Bierbeek, Belgium).

2.3. GC system

The column oven program was run at 120°C for 0.5 min, then heated with a temperature rate of 30°C/min to 300°C and held isothermically for 6.5 min, the complete run requiring 13 min. The detector temperature was 320°C. The TSD bead probes worked in the current range of 2.9 to 3.0 A, depending on the individual bead. The injector temperature was 300°C and a splitless injection mode was used. The split valve was opened after 0.5 min with a split ratio of 1:25 and the gas flow-rates were as follows: carrier gas (helium, 5.0): 3 ml/min (0.5 bar column head pressure), make up gas (nitrogen): 25 ml/min, hydrogen: 3.5 ml/min, synthetic air: 175 ml/min.

2.4. Bead characteristics

In order to overcome the short life-span of the original Varian TSD bead, which is extremely sensitive to moisture, acetone and silicone compounds, we used a TSD bead consisting of a rubidium dotted glass phase that is more stable and exhibits a higher analytical sensitivity than the Varian bead. Thus, we were able to measure approximately 400–500 samples using one bead.

2.5. Standard solution

Different standard dilutions of roc, 17OH-roc and 3OH-vec were prepared from three stock solutions, each containing approximately 1 mg of the respective compound, dissolved in 10 ml 0.8 *M* sodium dihydrogenphosphate. The pH was adjusted to 5.4 with 5 *M* NaOH. Standard dilutions were prepared monthly to overcome a concentration process and microbial contamination due to repeated opening of the flask. The standards were stable at 4°C for at least 3 months when stored in closed vessels.

2.6. Sample treatment

Heparinized blood was centrifuged (2500 U/min) for 5 min. To prevent hydrolysis of roc, the decanted plasma was diluted with an equal volume of 0.8 *M* phosphate buffer (pH 5.4) prior to storage. Repeated measurements of roc concentration–time courses of single patients, which showed good comparability with determinations of the same samples carried out approximately one year ago, confirmed the stability statement for roc from Kleef et al. [7].

2.7. Calibration curve

The calibration curves were generated by spiking drug-free heparinized plasma with between five and seven different amounts of roc and 17OH-roc (see Section 2.8). These standard samples were subjected to the same procedure as the patient samples. This was necessary because the slopes of the calibration curves produced from direct derivatisation of the pure compounds dissolved in acetonitrile or extracted from phosphate buffer solution were different from those resulting from the extraction of spiked plasma (see Section 2.10). Drug-free heparinized plasma was obtained from healthy coworkers and acidified with phosphate buffer to pH 5.4 (see Section 2.6).

2.8. Sample preparation

Aliquots (500 μl) of a drug-free sample/buffer mixture (250 μl plasma) were spiked with 0.6 (for roc), 1, 5, 10, 20, 40, 80 μl of a stock solution of roc or 17OH-roc, equivalent to 50, 80, 400, 800, 1600, 3200 and 6400 ng/ml plasma. Standard samples were treated in the same manner as patient samples. The calibration graphs were constructed by applying linear regression on the data of the peak area ratio analyte/internal standard and the amount of the analyte. The reliability of the used calibration curve was tested after measurement of 20 to 30 samples, taken from one patient during surgical intervention, with spiked plasma samples at three different concentrations. Deviations of more than 20% from the calculated amount of roc and 17OH-roc led to a recalibration of the system, a procedure which was routinely performed after approximately 40–60 samples. Spiked plasma for estimation of precision and accuracy was prepared in the same way as spiked plasma for the calibration curve. Aliquots (600 μl) of different concentration ranges were stored at −70°C and were stable for at least one year.

Measurement of these aliquots over a 12 months period were included in the inter-assay precision and the accuracy determination.

2.9. Extraction procedure

2.9.1. Liquid–liquid extraction with dichloromethane using KI as the ion-pairing reagent

A screw-capped tube was charged with 500 μl patient sample (250 μl plasma mixed with 250 μl phosphate buffer), 500 μl of saturated aqueous KI solution and 5 ml dichloromethane, the upper liquid phase after extraction. After 30 min extraction on a rotary mixer, the dichloromethane phase was decanted and evaporated at 60°C in a Speed Vac. The residue was dissolved in 200 μl acetonitrile and derivatized overnight with MTBSTFA at 70°C. Subsequently, acetonitrile and volatile byproducts were evaporated in a Speed Vac. The derivatized compounds were dissolved in 100 μl acetone where they are stable for at least one week at 4°C. 2 μl of these probes were injected into the gas chromatograph.

2.9.2. Ion-paired extraction with extrelut cartridges

Ion-paired extraction supported by Extrelut cartridges proved an adequate alternative to the above mentioned extraction method. A pre-packed column closed with a luer lock at the column end was charged with 1 ml sample mixture (500 μl patient sample and 500 μl saturated KI solution). After 5 min, 5 ml dichloromethane were applied and left for 5 min in order to equilibrate the mixture. After removing the luer lock the eluting water-free organic solvent was collected in a glass tube. The dichloromethane was evaporated, the residue dissolved in acetonitrile and subsequently derivatized with MTBSTFA as described in Section 2.9.1.

2.10. Assay validation

Assay validation and measurement of patient samples were performed using the first dichloromethane liquid–liquid extraction with KI as the ion-pairing agent. The intra-assay precision was assessed by measuring each of two control samples five times, the controls having been spiked with different concentrations of roc and 17OH-roc (400, 1052 ng/ml). The inter-assay precision (between-day variation) was calculated by measuring pooled plasma spiked with roc and 17OH-roc in the range of 400 to 1480 ng/ml over 15 days (Table 1).

The accuracy was expressed as the mean measured concentration and the mean found percentage of the added amount, together with the C.V. (%) (Table 2). The recoveries of the compounds from spiked plasma and from spiked phosphate buffer were determined, comparing peak area ratios of different concentrations (calibration standards) with peak area ratios of the compounds dissolved in acetonitrile, derivatized and quantified without prior extraction. The peak area ratios roc/I.S. and 17OH-roc/I.S. found in the standard acetonitrile solutions were referred to the calculated concentrations and defined as 100% recovery (Table 3). The same results were obtained when comparing the slopes of the different calibration curves (see Section 2.7). The amount of the compounds resulting in a signal-to-noise ratio

Table 1
Intra- and inter-assay precision for roc and 17OH-roc in spiked plasma

	Roc		17OH-roc	
	Mean concentration (ng/ml)	C.V. (%)	Mean concentration (ng/ml)	C.V. (%)
Intra-assay precision				
400 ng/ml, $n=5$	417	9.7	370	13.2
1052 ng/ml, $n=5$	1264	10.5	980	15.3
Inter-assay precision				
400 ng/ml, $n=15$	353	13.3	483	16.4
800 ng/ml, $n=13$	796	17.7	864	21.3
1480 ng/ml, $n=15$	1522	8.2	–	–

Table 2
Accuracy of the method for roc and 17OH-roc in spiked plasma

	Roc			17OH-roc		
	Mean concentration (ng/ml)	Found (%)	C.V. (%)	Mean concentration (ng/ml)	Found (%)	C.V. (%)
400 ng/ml, $n=15$	353	88	9.7	483	121	20.3
800 ng/ml, $n=13$	796	99	17.7	864	108	21.3
1480 ng/ml, $n=15$	1522	103	8.2	–	–	–

ranging from 5 to 10 (depending on the respective glass phase bead) was defined as lower limit of detection. The lower limit of quantification was estimated from repeated reliable determination of the lowest calibration standards.

3. Results and discussion

3.1. Results

The intra- and inter-assay C.V.s (%) are summarized in Table 1, ranging from 8–18% and 13–21% for roc and 17OH-roc, respectively. In order to produce a reliable between-patient comparability, recalibration of the system was carried out after the measurement of approximately 40–60 samples, equal to the time course measurement of roc concentrations from two patients. The mean accuracy ranged from 88–103% and from 108–121% for roc and 17OH-roc, respectively (Table 2).

The calibration curve is linear from 50–6400 ng/ml for roc and 80–6400 ng/ml for 17OH-roc according to the equation $y=a+bx$, with y being the spiked amount of roc and 17OH-roc and x being the ratio of roc/I.S. and 17OH-roc/I.S., respectively. Slopes of the calibration curves ranged from 1043–1166 and from 1163–1423 for roc and 17OH-roc, respectively. The constant term a ranged from 2–10 ng/ml for roc and 2–20 ng/ml for 17OH-roc, the correlation coefficient ranged from 0.980–0.999 for both compounds.

Table 3
Recoveries for roc, 17OH-roc and 3OH-vec in spiked phospate buffer and spiked plasma

	Phosphate buffer		Heparinized plasma	
	Found (%)	C.V. (%)	Found (%)	C.V. (%)
roc	73	15	48	13
17OH-roc	52	17	30	12
3OH-vec	102	11	97	9

Recoveries for roc and 17OH-roc in buffer and plasma were calculated in relation to the recoveries of the compounds dissolved in acetonitrile, derivatized and measured without prior extraction procedure (defined as 100%).

3.1.1. Extraction efficiency

Despite the poor recoveries of roc achieved with our extraction method (Table 3), we succeeded in detecting low roc concentrations even in patient samples 4 h after the final roc infusion (Fig. 2). Due to the high sensitivity of the glass phase bead of the nitrogen sensitive detector, the detection limit was 10 ng/ml for roc (50 pg absolute) and 50 ng/ml for 17OH-roc (250 pg absolute). In accordance with Kleef et al. [7], we were unable to detect 17OH-roc, the putative metabolite of roc, in any of the patient samples.

We attempted to improve the recovery data for roc and 17OH-roc for our analytical system, but neither the use of alternative organic extraction solvents (1,1,1-trichloroethane, trichloromethane, diethylketone and ethylmethylketone) with and without KI, nor the application of solid-phase extraction methods using RP8, RP18, cyanopropyl or cation-exchange cartridges resulted in improved recoveries. Recoveries of the three compounds resulting from CH_2Cl_2/KI ion-paired extraction supported by Extrelut pre-packed glass columns were similar to normal liquid–liquid extraction. The advantages of Extrelut extraction are the shorter extraction time (5–10 min compared to 30 min) and the water-free separated organic phase, avoiding the time consuming separation of CH_2Cl_2 from the aqueous phase after normal liquid–liquid extraction. Despite these

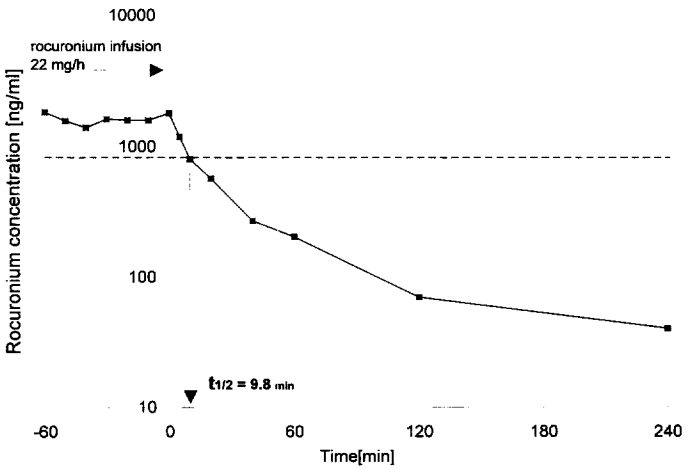

Fig. 2. Roc plasma levels of a 45 yr old woman (165 cm, 45 kg). First part (−60 to 0): blood samples taken every 10 min during permanent i.v. infusion (22 mg/h) over 90 min, a 95% neuromuscular blockade was maintained; descending part (0 to 240): monitoring of roc concentrations for 4 h after end of infusion.

advantages we decided to use the initial extraction method because of the reduced outlay.

No analytical interferences occurred in the chromatograms of plasma free of roc, 17OH-roc and 3OH-vec (Fig. 3).

3.2. General comments

Desallylrocuronium, an additional putative metabolite of roc (Fig. 1) was not detectable with the cGC method because the nitrogen atom at the 16 steroidal position of roc thermically dequarternizes in the injection port forming desallylrocuronium. Thus, the two compounds cannot be distinguished from each other. Kleef et al. [7] have demonstrated that desallylrocuronium could be separated from the other compounds, but was not detectable in patient samples.

3.3. Pharmacokinetics

The pharmacokinetic analysis enables the discussion of possible reasons for the different infusion rates, such as decreased volume of distribution and decreased plasma clearance in the steady state. Infusion rates maintaining a 95% neuromuscular blockade were significantly different in obese and asthenic patients when compared to patients with normal weight. First results, to be published elsewhere [8], show a comparable neuromuscular recovery in patients with different BMI (4 BMI: <21; 21–26; 26–30; >30) and a normal context sensitive

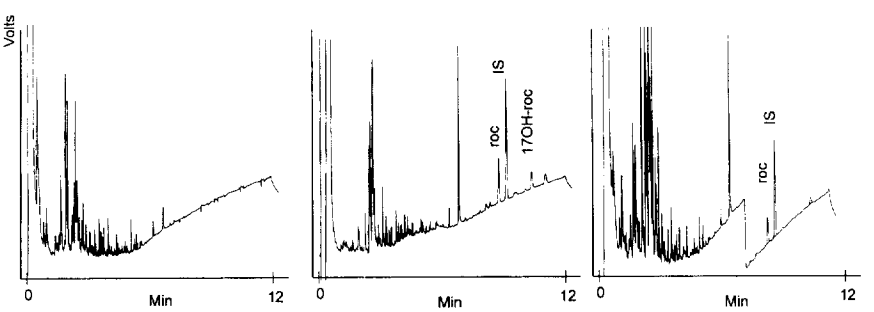

Fig. 3. Representative chromatograms of drug-free plasma, spiked plasma (c=1132 ng/ml for roc and 17OH-roc) and a patient sample (c=1092 ng/ml for roc, 17OH-roc not detectable).

half life time (9.8 min) in obese patients compared to patients with normal weight. Fig. 3 shows an example for roc plasma levels of a female patient during roc infusion and up to 4 h after infusion stop.

4. Conclusions

A reliable and easy-to-use cGC-method has been developed and was applied to the pharmacokinetic study of roc in patients with normal weight and with extreme habitus. For reasons of sensitivity and specificity it was not possible to use a more robust flame ionisation detector. With the rubidium dotted glass phase bead of the nitrogen specific detector a reliable and accurate determination was possible. The use of Extrelut cartridges simplifies the extraction procedure and saves valuable sample preparation time in large scale determinations.

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