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Determination of rocuronium and its putative metabolites in body fluids and tissue homogenates

U. W. Kleef*

Research Group for Experimental Anesthesiology and Clinical Pharmacology, University Hospital, Oostersingel 59, 9713 EZ Groningen (Netherlands)

J. H. Proost

Department of Pharmacology and Therapeutics, University Centre for Pharmacy, University of Groningen, Antonius Deusinglaan 2, 9713 AW Groningen (Netherlands)

J. Roggeveld

Research Group for Experimental Anesthesiology and Clinical Pharmacology, University Hospital, Oostersingel 59, 9713 EZ Groningen (Netherlands) and Department of Pharmacology and Therapeutics, University Centre for Pharmacy, University of Groningen, Antonius Deusinglaan 2, 9713 AW Groningen (Netherlands)

J. M. K. H. Wierda

Research Group for Experimental Anesthesiology and Clinical Pharmacology, University Hospital, Oostersingel 59, 9713 EZ Groningen (Netherlands)

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ABSTRACT

A sensitive and selective HPLC method was developed for the quantification of the neuromuscular blocking agent rocuronium and its putative metabolites (the 17-desacetyl derivative and the N-desallyl derivative of rocuronium) in plasma, urine, bile, tissue homogenates and stoma fluid. Samples were prepared by extraction of the biological matrix with dichloromethane, after mixing with a KI–glycine buffer. After evaporation of the organic solvent the samples were chromatographed on a reversed-phase HPLC column, using an aqueous buffer–dioxane (84:16, v/v) as the mobile phase. The aqueous buffer consisting of 0.1 *M* sodium dihydrogen phosphate, 0.11 m*M* 9,10-dimethoxyanthracene-2-sulphonate (DAS), 0.11 m*M* 1-heptane-sulfonic acid, was adjusted to pH 3 with orthophosphoric acid. After separation, the eluent was extracted with dichloroethane, and the organic phase was led to a fluorimetric detector, operating at 385 nm (excitation) and 452 nm (emission). The method was validated for the assay in plasma, urine, bile, tissue homogenates and stoma fluid, by determination of the repeatability, reproducibility, accuracy, lower limit of quantification, lower limit of detection, extraction recovery, effect of sample volume, and stability in the biological matrix. The method was found to be sensitive (lower limit of quantification for rocuronium in plasma is 10 ng/ml) and accurate. The interference of concomitant drugs with the assay of rocuronium and its putative metabolites has been studied extensively. In order to confirm the identity of rocuronium and its putative metabolites, a TLC method was developed. The method has been applied successfully in several pharmacokinetic studies with rocuronium.

* Corresponding author.

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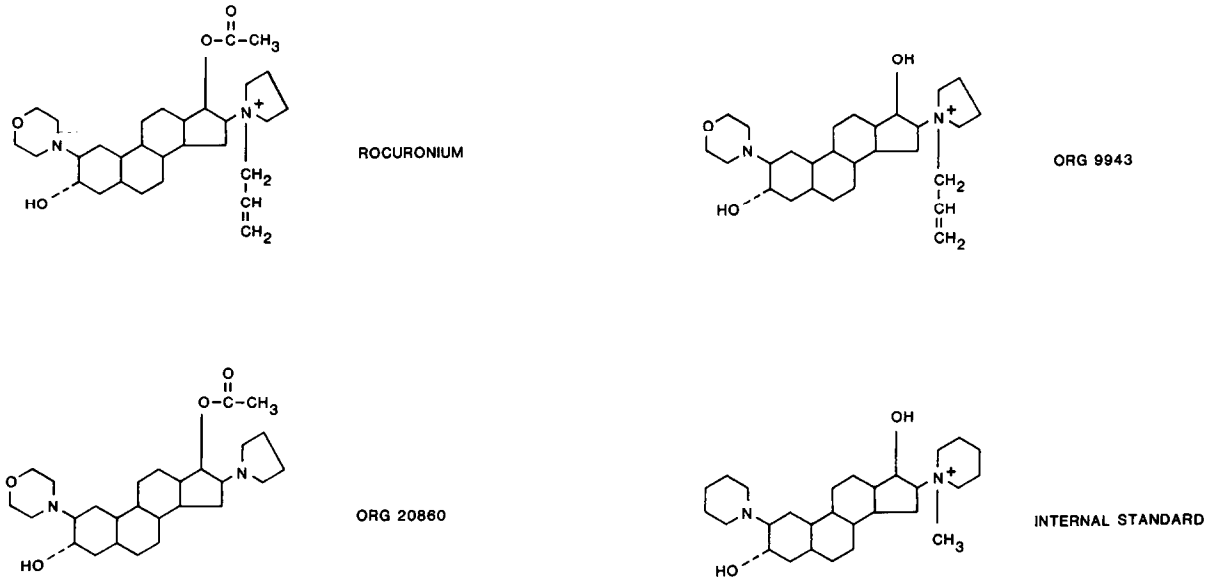

Fig. 1. Structural formulas of rocuronium, its two derivatives and the I.S. (Org 7402).

INTRODUCTION

Rocuronium (Org 9426) (see Fig. 1) is a new aminosteroidal nondepolarizing neuromuscular blocking agent, characterized by a rapid onset of action and a duration of neuromuscular blockade comparable to that of the related compound vecuronium [1,2]. In order to study the pharmacokinetics of a (new) drug it is necessary to have the disposal of a reliable, accurate, sensitive, rapid and economic analytical method for the determination of the concentrations of the compound and its putative metabolites in body fluids and tissue homogenates, which can discriminate between the compounds of interest and other drugs administered simultaneously.

For vecuronium a sensitive assay in plasma was developed by Paanakker *et al.* [3], using solid-phase extraction, followed by separation by HPLC, post-column ion-pair extraction with the fluorescent counter-ion 9,10-dimethoxyanthracene-2-sulphonate (DAS) and fluorimetric detection. Recently, two new HPLC methods for the bioanalysis of vecuronium have been published, using conductivity [4] and electrochemical [5] detection. An extensive review of the analytical methods used for determination of neuromuscular blocking agents in man can be found in ref. [6]. However, no specific assay for rocuronium and its putative metabolites, applicable for routine and large-scale determinations, has been described. In order to meet the aforementioned requirements, the HPLC method for vecuronium, described by Paanakker *et al.* [3], was modified for rocuronium and its putative metabolites Org 9943 (17-desacetyl Org 9426) and Org 20860 (N-desallyl Org 9426).

In order to confirm the identity of a peak in a chromatogram, a second, different separation system must be applied, in particular in clinical pharmacokinetic studies where concomitant drugs and their metabolites may interfere with the analysis of the compounds of interest. Therefore we developed the qualitative thin-layer chromatographic (TLC) method to be used in combination with the quantitative HPLC system.

The aim of the work was to develop a method for the routine analysis of rocuronium and its putative metabolites, and to validate the method for the application in pharmacokinetic studies with respect to repeatability, reproducibility, accuracy, influence of sample dilution, lower limit

of quantification, lower limit of detection, stability of the compounds in a biological matrix, and the interference of drugs commonly applied perioperatively.

EXPERIMENTAL

Chemicals

Rocuronium bromide, its two putative metabolites (Org 9943, the 17-desacetyl derivative of rocuronium, and Org 20860, the N-desallyl derivative of rocuronium) and the internal standard (Org 7402, the 3,17-dihydroxy derivative of vecuronium) were supplied by Organon Laboratories (Newhouse, Scotland) and Organon Teknika (Boxtel, the Netherlands). Methanol, 1,4-dioxane, dichloromethane, 1,2-dichloroethane and 2-propanol were of *pro analysi* quality (Merck, Darmstadt, Germany). 9,10-Dimethoxyanthracene-2-sulphonate (DAS), obtained from Fluka AG (Buchs, Switzerland), was subjected to soxhlet extraction with dichloroethane before use. NaH_2PO_4, orthophosphoric acid (35%), NaCl, NaOH, glycine, KI, NaI and hexachloroplatin III acid solution were of *pro analysi* quality from Merck. 1-Heptane-sulfonic acid sodium salt 98% was obtained from Janssen Chimica (Goirle, the Netherlands). Thin-layer silicagel plates (DC Fertigplatten, 20 × 20) were obtained from Whoelm Pharma (Eschwege, Germany). Water was of Millipore Q-quality.

Stock and standard solutions

Stock solutions (freshly prepared each month) of all reference compounds (1 mg/ml) were prepared in 0.1 *M* NaH_2PO_4 adjusted to pH 3.0 with orthophosphoric acid. Working solutions for spiking were prepared weekly by dilution with 0.1 *M* NaH_2PO_4 (pH 3.0) to a final concentration of 100 or 10 μg/ml.

KI–glycine buffer

KI–glycine buffer was prepared from 6 ml of 0.1 *M* glycine buffer (0.7505 g glycine, 0.585 g NaCl made up with distilled water to 100 ml), 4 ml of 0.1 *M* NaOH and 6.2 g of KI.

Iodoplatinate reagent

Three ml of the hexachloroplatin solution were diluted up to 100 ml with distilled water and mixed with 100 ml of a 6% KI solution in distilled water.

Instrumental

Waters Model 510 HPLC pumps (Millipore Waters, Milford, MA, USA) were used as the HPLC solvent delivery pump and as the post-column pump for the delivery of the organic extractant. A Model 712 WISP autosampler was obtained from Millipore Waters.

The phase separator was obtained from Organon International (Oss, the Netherlands) [1]. It consists of two stainless-steel blocks, one with a groove and one without a groove, and a PTFE disc with a groove, sandwiched together. As a result a groove is obtained, the upper half being of stainless steel and the lower half of PFTE with a total internal volume of *ca.* 30 μl. The aqueous outlet (HPLC eluent and excess of organic extractant) is connected to a microcontrol needle valve SMVC (SQE, Ringwood, Australia) to allow the application of back-pressure to adjust the organic flow through the fluorimetric detector. A Model 470 fluorimetric detector and the integrator (Waters 741 data module) were obtained from Millipore Waters.

Acidification and storage of samples

To prevent possible hydrolysis of rocuronium, human plasma, urine and bile were acidified to a pH below 6 by addition of 200 μl of 1 *M* NaH_2PO_4 per ml of sample. After dissection tissues were carefully blotted dry and homogenized for 10 min with 1 *M* NaH_2PO_4 in a ratio 1:9 (1 g tissue/9 ml NaH_2PO_4) by using an ultra-turax. Stoma fluid was homogenized with 200 μl of 1 *M* NaH_2PO_4 per ml stoma fluid. Samples were stored at −20°C until analysis.

Extraction procedure

In a glass-stoppered tube the biological samples (plasma 50–1000 μl, urine 200–1000 μl, bile 5–200 μl, homogenates 100–1000 μl and stoma fluid 1000 μl) were made up to *ca.* 2.0 ml with

demineralized water. KI–glycine buffer (1 ml) and 150 ng of the internal standard were added. The mixture was shaken for 15 s on a vortex mixer with 7.0 ml of dichloromethane. After centrifugation for 5 min (at 740 *g*) the upper phase was discarded and the organic layer was evaporated to dryness under a stream of air at 37°C. The residue was dissolved in 200 μl of mobile phase and a volume of 100 μl or less was introduced onto the HPLC system, using the WISP autosampler.

HPLC system

The HPLC system was similar to the system described for vecuronium [3]. A Lichrosopher 100-RP18 (5 μm) column (150 × 39 mm I.D.) and a μBondapack C_{18} precolumn (4 × 6 mm I.D.) were used. The eluent consisted of water–dioxane (84:16, v/v). The water contained 0.1 *M* NaH_2PO_4, 0.11 m*M* DAS and 0.11 m*M* 1-heptane-sulfonic acid, adjusted to pH 3.0 with orthophosphoric acid. The eluent was degassed in an ultrasonic bath and pumped at a flow-rate of 1.0 ml/min. The organic extractant was dichloroethane and the flow-rate was 1.2 ml/min.

The low-dead-volume T-piece was made of stainless steel. The capillary coil was made of stainless steel (1 m × 0.25 mm I.D.) with a coil diameter of approximately 9 mm.

The splitting ratio after phase separation was approximately 30% (= organic flow through the detector: aqueous/organic flow to waste). The fluorimetric detector was operated at 385 nm (excitation) and 452 nm (emission).

After each series of analysis the HPLC column was flushed with water (*ca.* 15 ml) and methanol (*ca.* 75 ml). The chromatographic system was operated at room temperature.

Calibration

Samples for calibration were prepared freshly by adding known amounts of rocuronium and its metabolites to drug-free plasma (200 μl), urine (200 μl), bile (100 μl), liver homogenate (100 μl), lung homogenate (1000 μl) and stoma fluid (1000 μl). The samples were submitted to the procedure described.

Calibration graphs were constructed by applying linear regression on the logarithmically transformed data of peak-height ratio analyte/internal standard (response ratio, *RR*), and the amount of analyte, according to the following hypothesized relationship:

$$RR = a \times X^b$$

or, after logarithmic transformation:

$$\log RR = \log a + b \log X$$

where *RR* is the ratio of the peak heights of the compound to be analyzed and the internal standard (I.S.); *X* is the known amount added to the sample. The constants *a* and *b* were obtained by linear regression analysis of the log-transformed data of each calibration line.

The amounts of Org 9426, Org 9943 and Org 20860 in the unknown samples (*Y*) were calculated from

$$Y = \text{antilog}\left(\frac{\log RR - \log a}{b}\right)$$

where *a* and *b* were obtained from the calibration line as described above. The unknown samples were calculated using calibration curves obtained on the same day.

Thin layer chromatography

Up to 5 ml of the biological samples were extracted in the same way as described before. The residue of the organic phase was dissolved in 0.05 ml of 0.01 *M* HCl which was applied to the thin layer plate. The plates were developed in a 2% solution of NaI in 2-propanol and were allowed to run for about 4 h. The plates were dried and coloured by spraying with iodoplatinate reagent.

Interference of drugs in the HPLC assay

The compounds screened in the assay were first introduced onto the HPLC system as pure standards. Compounds which were found to give a response were added to drug-free biological samples and to spiked samples and were submitted to extraction and HPLC.

Assay validation

Blank human plasma was obtained from a number of volunteers, pooled, partly acidified with 1 *M* NaH_2PO_4 in a ratio 5:1, and partly unacidified. Plasma samples from patients collected immediately before administration of rocuronium were analyzed and pooled.

Blank human urine was obtained from healthy volunteers and acidified with 1 *M* NaH_2PO_4 in a ratio 5:1.

Blank human bile was collected from patients with temporarily extracorporeal bile drainage due to extrahepatic tract obstruction. The bile was acidified with 1 *M* NaH_2PO_4 in a ratio 5:1.

Blank stoma fluid was obtained from 4 different patients with a permanent ileo stoma. The stoma fluid was homogenized with 1 *M* NaH_2PO_4 in a ratio 5:1. The stoma fluids were analyzed and pooled.

Blank plasma, urine, bile, and liver and lung homogenates of the dog were supplied by Organon (Schayk, the Netherlands). The plasma, urine and bile were not acidified. The tissues were homogenized with 1 *M* NaH_2PO_4 in a ratio 1:9. All blank biological materials were divided into a number of tubes and stored at −20°C until analysis.

The intra-day precision (repeatability) was assessed using replicate ($n = 5$) calibration samples and is expressed in the coefficient of variation (C.V.).

The inter-day variability (reproducibility) was calculated from calibration curves obtained over an extended period of time by three different analysts. The inter-day variability is expressed in the coefficient of variation.

The accuracy of the assay was investigated by manifold analyzing drug-free samples spiked with the compounds in a range of concentrations. The samples were assayed blindly and the concentrations were derived from calibration curves prepared on the same day. The accuracy is expressed as percentage found of the added amount.

The extraction recovery from plasma was determined by comparing the peak heights for each compound and I.S. in extracted standards with those obtained by injection of unextracted standards.

The effect of sample volume on the extraction recovery was studied by analyzing samples containing various amounts of plasma (10–1000 μl), urine (5–1000 μl), bile (5–100 μl), stoma fluid (5–1000 μl) and the same amount of compounds (10, 25, 50, 200, 500 or 1000 ng).

The lower limit of quantitation (LOQ) was defined as the minimum concentration which could be detected with an accuracy and precision better than 15%. The lower limit of detection (LOD) was defined as the amount of compound resulting in a signal-to-noise ratio of 5.

The stability of the compounds was determined in the various biological samples stored at −20°C, at a pH below 6 (samples acidified with 1 *M* NaH_2PO_4) and at physiological pH. Drug-free human plasma, urine, bile and stoma fluid, containing NaH_2PO_4 and drug-free homogenates were spiked with either rocuronium or with the three compounds in a range of concentrations (plasma 10–10 000 ng/ml, urine 25–100 000 ng/ml, bile 233–76 923 ng/ml, stoma fluid 100–4000 ng/ml and tissue homogenates 25–500 ng/ml). The stability at physiological pH was investigated in spiked plasma and urine of the dog and in human plasma. The samples were analyzed in duplicate at various times after storage at −20°C over a long period of time (2.5–14 months, see Results). The concentrations were calculated using calibration curves obtained on the day of the analysis.

RESULTS AND DISCUSSION

Representative chromatograms of extracts of blank, spiked and clinical samples of human plasma, urine, bile and stoma fluid are shown in Fig. 2. No interfering peaks of endogenous substances and of compounds used for induction and maintenance of anesthesia appear in the chromatographs of plasma, urine and bile. From the chromatograms of the 4 different blank stoma fluids it was obvious that the desallyl derivative, Org 20860, indeed could be detected but not reliably quantitated, due to endogenous substances. Sep-

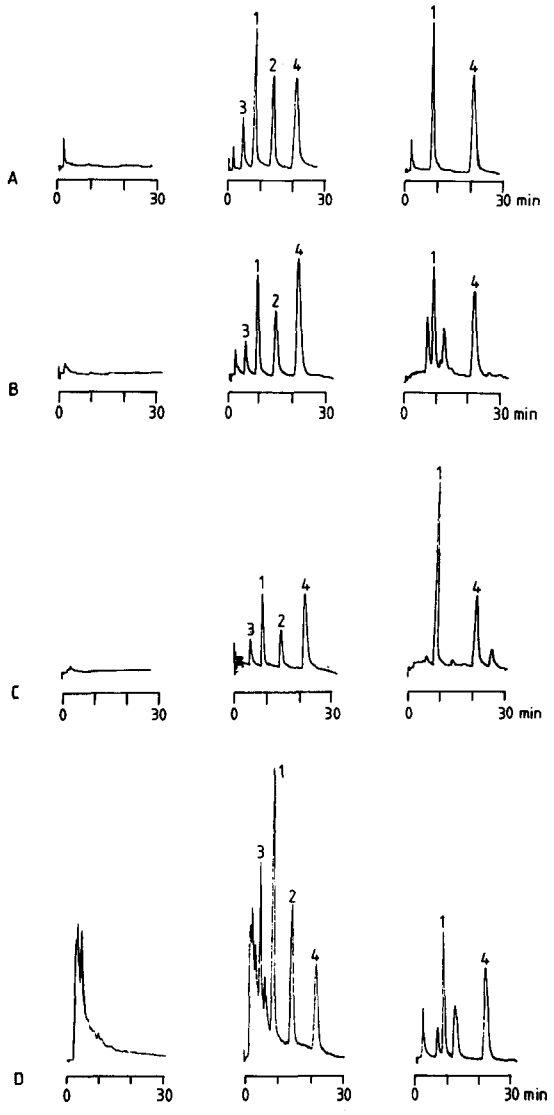

Fig. 2. Chromatograms of human samples.
(A) Left: plasma sample, collected from a patient before the administration of rocuronium. Middle: blank plasma spiked with 250 ng of rocuronium (1), Org 9943 (2), Org 20860 (3) and 150 ng of I.S. (4). Right: plasma sample collected from a patient 10 min after the administration of rocuronium.
(B) Left: blank urine from a patient. Middle: blank urine spiked with 125 ng of rocuronium (1), Org 9943 (2), Org 20860 (3) and 150 ng of I.S. (4). Right: urine sample collected from a patient 12–18 h after the administration of rocuronium.
(C) Left: blank bile from a patient. Middle: blank bile spiked with 150 ng of rocuronium (1), Org 9943 (2), Org 20860 (3) and I.S. (4). Right: bile sample collected from a patient 18–24 h after the injection of rocuronium.
(D) Left: blank stoma fluid. Middle: blank stoma fluid spiked with 500 ng of rocuronium (1), Org 9943 (2), Org 20860 (3) and 150 ng of I.S. (4). Right: stoma fluid collected from a patient 14 h after the injection of rocuronium.

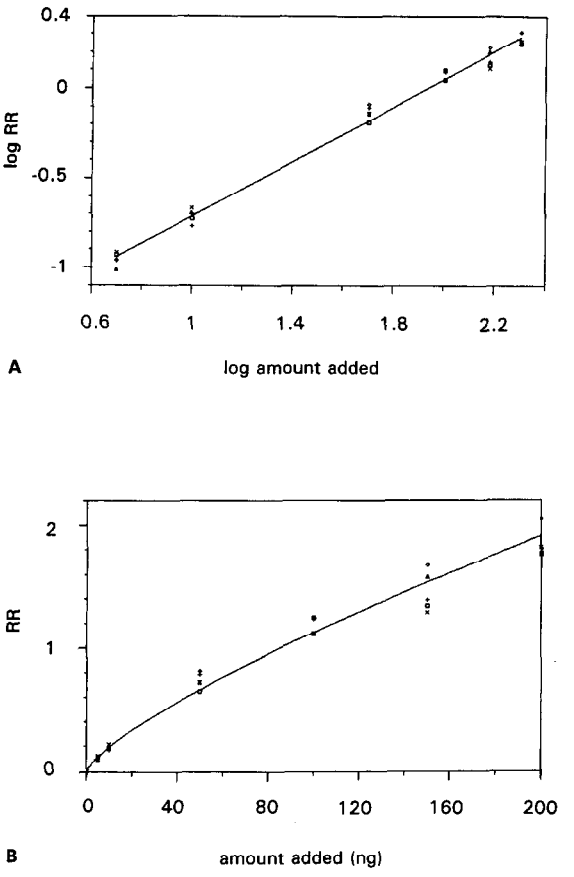

Fig. 3. Calibration curves in human plasma both in a double logarithmic (A) and in a linear plot (B). Each symbol represents a single measurement (n = 5).

aration of Org 20860 and the interfering compounds could not be obtained by adapting the flow and the concentrations of DAS and/or dioxane in the eluent.

For each of the compounds and in each matrix the relationship between the added amount and the response ratio was found to be curvaceous. After logarithmic transformation of both axes, linear calibration curves were obtained for each of the compounds and in each matrix (Fig. 3).

The results of the validation have been summarized in Tables I (intra-day variability), II (inter-day variability) and III (accuracy) for human plasma, urine, bile and stoma fluid and dog liver homogenate. Comparable results were obtained for dog plasma, urine and bile, and for dog lung tissue homogenate (data not shown).

The intra-day precision of the three compounds appeared to be almost the same for each matrix and was only slightly dependent on the amount of compound (Table I). As indicated by the C.V. the mean values were 6%, 8% and 6% for rocuronium, Org 9943 and Org 20860, respectively. Almost similar values were found in the assayed accuracy samples (Table III). The mean values for the C.V. were 6%, 7% and 9% for rocuronium, Org 9943 and Org 20860, respectively.

The inter-day variability (shown in Table II)

TABLE I

INTRA-DAY VARIABILITY IN HUMAN PLASMA, URINE, BILE AND STOMA FLUID, AND IN DOG LIVER HOMOGENATE

Added amount (ng)	*RR* rocuronium		*RR* Org 9943		*RR* Org 20860	
	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
Plasma (n = 6/5)						
10	0.147	5.7				
20	0.241	5.5	0.139	9.0	0.081	18.6
50	0.485	6.3	0.291	12.9	0.234	6.9
100	0.822	9.7	0.497	8.8	0.376	11.1
200	1.651	5.7	0.999	9.7	1.021	10.2
500	2.330	3.9	1.582	8.1	1.800	7.8
1000	4.677	5.1	2.717	8.4	4.090	5.9
Urine (n = 5)						
25	0.306	1.8	0.202	8.9	0.125	12.8
50	0.478	10.9	0.322	9.2	0.222	6.1
100	0.831	6.8	0.529	2.8	0.388	8.1
250	1.529	9.0	0.987	1.0	0.824	10.0
500	2.412	8.2	1.551	5.1	1.499	7.9
1000	3.827	8.0				
Bile (n = 5)						
10	0.127	12.4	0.095	12.5	0.096	6.7
25	0.336	15.8	0.163	14.6	0.197	4.5
50	0.558	5.1	0.278	13.6	0.365	4.0
100	0.949	7.1	0.537	4.2	0.636	4.8
250	1.999	5.4	1.106	4.6	1.497	4.2
500	3.411	4.4	1.706	4.9	2.769	3.4
1000	6.326	9.2				
Stoma fluid (n = 4)						
20	0.278	2.4	0.097	8.3	Not quantifiable	
100	1.006	2.5	0.456	7.4	Not quantifiable	
250	1.906	4.7	0.886	1.0	Not quantifiable	
500	3.228	4.9	1.442	3.5	Not quantifiable	
1000	5.425	2.0	2.410	3.1	Not quantifiable	
Liver homogenate (n = 5)						
10	0.158	6.3	0.086	6.3		
25	0.323	6.1	0.187	5.6	0.095	19.6
100	0.896	5.4	0.521	5.0	0.343	7.0
250	1.693	4.6	0.991	5.0	0.798	7.9
500	2.604	1.6	1.552	2.9		
1000	4.774	0.8	2.720	1.7	3.590	7.3

TABLE II

INTER-DAY VARIABILITY IN HUMAN PLASMA, URINE, BILE AND STOMA FLUID

Added amount (ng)	*RR* rocuronium			*RR* Org 9943			*RR* Org 20860		
	Mean	C.V. (%)	n^a	Mean	C.V. (%)	n^a	Mean	C.V. (%)	n^a
Plasma									
10	0.129	15.2	45	0.073	25.2	11	0.063	21.9	12
50	0.463	13.5	54	0.307	16.5	26	0.207	20.8	14
100	0.879	15.8	54	0.519	17.3	25	0.410	30.5	15
250	1.660	12.5	53	1.171	17.5	25	0.904	35.4	15
1000	4.983	13.5	54						
Urine									
25	0.301	15.2	8	0.199	7.1	7	0.143	18.4	7
100	0.961	12.8	12	0.569	13.7	10	0.467	25.9	10
250	1.772	13.7	11	1.091	12.8	9	1.045	20.0	10
500	2.955	21.3	12	1.849	18.7	8	2.180	19.3	7
1000	5.303	20.0	9	3.531	21.2	6	5.069	14.2	5
Bile									
10	0.153	16.4	12	0.077	21.3	9	0.068	29.1	5
25	0.333	14.6	14	0.174	18.5	11	0.136	23.7	9
100	1.031	11.7	14	0.523	15.2	11	0.435	18.4	9
500	3.249	16.3	14	1.691	15.5	11	2.033	21.4	7
1000	5.062	13.1	11	2.839	15.5	10	3.904	11.0	5
Stoma fluid									
20	0.304	8.7	3	0.128	12.9	3	Not quantifiable		
100	1.131	16.4	4	0.494	16.3	4	Not quantifiable		
250	2.307	19.1	4	1.029	16.8	4	Not quantifiable		
500	3.960	21.6	4	1.792	17.3	4	Not quantifiable		
1000	6.797	24.6	4	3.121	17.8	4	Not quantifiable		

[a] Number of samples analyzed over a period of 12 months (plasma, bile), 2.5 months (urine), and 2 months (stoma fluid).

was found to be relatively large when compared to the intra-day variability (15% for rocuronium, 17% and 22% for Org 9943 and Org 20860, respectively).

Since a coefficient of variation of the analytical assay higher than 15% contributes significantly to the overall variability of pharmacokinetics [7], the concentrations of unknown samples were calculated using calibration curves prepared on the day of the analysis. The calibration samples were randomly distributed among the unknown samples. The accuracy of blindly assayed samples, covering concentrations occuring in clinical samples are listed in Table III. The mean absolute deviation was found to be 5.6%, 6.9% and 9.2% for rocuronium, Org 9943 and Org 20860, respectively.

From the results of the intra-day variability (Table I) and the accuracy (Table III) the LOQ was found to be in plasma 10 ng/ml for rocuronium and 20 ng/ml for the two derivatives, in urine 25 ng/ml for rocuronium and Org 9943 and 50 ng/ml for Org 20860. In bile the LOQ was for rocuronium 100 ng/ml and 250 ng/ml for the two derivatives, whilst 20 ng/ml rocuronium and Org 9943 could reliably be determined in stoma fluid. In tissue homogenates the LOQ was 100 ng/ml for rocuronium and Org 9943, and 250 ng/ml for Org 20860.

The LOD was estimated to be 3, 5, 15 ng for rocuronium, Org 9943, Org 20860, respectively, in plasma, 4, 8, 10 ng in urine and bile, and 5, 10, and 25 ng in tissue homogenates.

TABLE III

ACCURACY IN HUMAN PLASMA, URINE, BILE AND STOMA FLUID, AND IN DOG LIVER HOMOGENATE

Concentration (ng/ml)	n	Sample volume (μl)	Rocuronium		Org 9943		Org 20860	
			Found (%)	C.V. (%)	Found (%)	C.V. (%)	Found (%)	C.V. (%)
plasma								
10	4	1000	97.0	3.4				
20	5	1000	92.0	7.9	91.5	10.3	92.6	6.9
40	5	1000	91.5	7.1	97.7	11.7	96.4	15.0
100	5	1000	96.5	4.0	94.2	6.3	102.3	6.3
200	5	1000	101.6	5.1	105.6	6.6	111.1	10.6
500	5	1000	94.5	8.7				
500	4	500	95.8	5.8				
1000	5	250	102.7	8.3				
1000	3	100	101.2	0.7				
5000	8	50	107.1	5.2				
10000	4	50	98.1	6.3				
urine								
25	5	1000	88.0	13.0	104.0	10.3	186.4	13.8
50	5	1000	88.0	4.4	98.0	3.3	116.5	7.6
236	5	500	100.4	10.4	102.5	10.1	96.6	6.8
250	4	500			113.6	7.7	102.8	6.6
1000	5	1000	101.1	4.6				
10000	5	50	96.7	4.4				
100000	5	5	108.4	14.1				
Bile								
100	5	100	99.4	1.7				
233	4	100	90.1	13.6	89.7	4.0	88.6	17.1
971	4	100			106.8	7.8	90.6	9.6
9709	4	25	98.9	3.3	103.3	8.4	98.8	9.1
76293	4	5	109.4	8.2	114.0	6.3	104.8	5.7
Stoma fluid								
20	4	1000	117.0	8.9	105.6	8.2	Not quantifiable	
120	4	1000	103.7	2.5	111.7	4.9	Not quantifiable	
500	4	1000	110.0	5.0	114.6	6.4	Not quantifiable	
997	4	1000	98.0	2.9	94.0	2.9	Not quantifiable	
Liver homogenate								
100	5	100	89.8	6.9	93.4	9.7		
250	5	100	102.8	5.1	106.2	7.7	85.0	8.3
500	5	100	97.3	2.3	100.7	3.5	91.4	13.2
2000	5	100	91.3	3.1	97.7	4.3	105.2	1.9
5000	5	100	93.7	1.8	95.4	3.4	92.2	9.1

Solid phase extraction, often used as sample preparation in the assay of related compounds [3,5] did not give an efficient recovery of the two derivatives from plasma, urine and bile, and is inadequate for tissue homogenates, since it does not allow the extraction of the compounds from the particle fraction. The extraction with excess of KI into dichloromethane, used for the extraction of pancuronium from biological materials [8], enabled processing of larger quantities of the

TABLE IV

DRUGS INTERFERING IN THE ASSAY OF ROCURONIUM AND ITS DERIVATIVES

Drug	Rocuronium	Org 9943	Org 20860	Internal standard
Antibiotics				
Cefotaxime			+	
Ceftazidime			+	
Ceftriaxone			+	
Cefuroxime			+	
Cephamandole			+	
Cephradine			+	
Tranquillizers				
Dixyrazine				+
Prochlorperazine	+		+	
Neuromuscular blocking agents				
Atracurium	+	+	+	
d-Tubocurarine	+			
3-Hydroxyvecuronium		+		
3-Hydroxypancuronium[a]		+		
Metocurine				+
Various				
Alizapride	+		+	
Co-trimoxazole			+	
Ketamine[a]	+			
Ketogan	+			
Lidocaine	+			
Metoclopramide	+	+	+	
Metoprolol				+
Nimodipine	+		+	
Pethidine[a]				+

[a] See text.

biological samples for application on TLC. The recoveries of the extraction from 1000 μl plasma were found to be equal for the three compounds and the I.S. in a range of 10–1000 ng. The mean (n = 3) recoveries were 91.5%, 90.6%, 91.6% and 90.3% for rocuronium, Org 9943, Org 20860 and the I.S., respectively. The amount of plasma did not affect the recovery of the compounds over a range of 50–1000 μl, which allowed the measurement of plasma concentrations of 10–20 000 ng/ml within the linearity of the calibration curves. No difference was found in the response ratio of rocuronium and Org 9943 after extraction from 5–1000 μl urine. The results for Org 20860 in 5 μl urine, containing 25 ng and 50 ng, appeared to deviate significantly. Therefore, if the analysis of urine samples required a volume less than 200 μl the volume was adjusted to 200 μl with blank urine. The recoveries of the compounds were not affected by the volume of bile ranging from 5–1000 μl, and by the volume of stoma fluid ranging from 100–1000 μl.

After 9.5 months storage at −20°C no degradation of rocuronium was found in plasma containing NaH_2PO_4 and in plasma at physiological pH. The recoveries ranged from 90.1 to 111.2% and from 96.6 to 104.0%, respectively. Acidified plasma spiked with the three compounds and stored for 14 months at −20°C showed a recovery of 96.6, 89.0 and 107.0% for rocuronium,

TABLE V

DRUGS NOT INTERFERING IN THE ASSAY

Anaesthetics	Analgetics	Neuromuscular blocking agents	Tranquillizers	Various
Etomidate	Alfentanil	Gallamine	Chlorpromazine	Aprotinin
Pentobarbital	Fentanyl	Pancuronium	Diazepam	Atropin
Propofol	Morphine	Pipecuronium	Droperidol	Bupivacaine
Thiopental	Sufentanil	Succinylcholine	Haloperidol	Dalteparin
		Vecuronium	Promethazine	Dexamethasone
				Dopamine
				Furosemide
				Midazolam
				Neostigmine
				Nitroglycerin
				Nitroprusside
				Oxytocin
				Phenylephrine
				Phenytoin
				Piperacilline
				Raniditine
				Terbutaline
				Verapamil

Org 9943 and Org 20860, respectively. In acidified urine, bile and tissue homogenates the compounds were stable for at least 2.5, 3.5 and 10 months, respectively. In urine samples at a pH between 6.0 and 7.2, stored at −20°C the stability of the compounds lasted for at least 3.5 months. Spiked stoma samples (20 ng/ml and 499 ng/ml) showed a recovery of 95.7% rocuronium at both concentrations, whilst for Org 9943 90.5 and 97.0%, respectively, was recovered after three weeks of storage at −20°C.

A problem often met in extensive pharmacokinetic studies and in toxicology is the interference of concomitant drugs and their possible metabolites in the assay of the compounds of interest. To confirm the identity of rocuronium and its metabolites found in the HPLC method, we developed a readily applicable TLC method. After spraying with iodoplatinate reagent (specific for N-containing organic compounds) the substances appeared as small brown spots on a light yellow background. The compounds were well separated with R_F-values of 31.8, 62.0 and 52.3 for rocuronium, Org 9943 and Org 20860, respectively. An amount of 500 ng of the compounds could be readily visualized. The support of a second qualitative separation method enhances the reliability of the results obtained from the quantitative HPLC method, especially in urine and bile, where the occurrence of metabolites of other administered drugs may cause an erroneous result. In case of doubt, *e.g.* due to limited sensitivity of the TLC method, the identity and purity of the chromatographic peaks and the occasional overlap with unknown substances may be assessed using HPLC combined with mass spectrometry.

A number of drugs screened in the assay as described are listed in Tables IV and V. The interference of pethidine, used to promote hypothermia by surface cooling in neurosurgical procedures, could be eliminated by lowering the eluent flow and the amount of dioxane to 15% in the eluent. The retention times for the I.S. and pethidine were 15.3 and 18.3 min., respectively. A separation of rocuronium and ketamine, often used in animal experiments, was achieved in the same way.

Lowering both the extractant and eluent flow

to 0.7 and 0.5 ml/min separated the 3-OH metabolite of pancuronium and Org 9943, allowing the administration of pancuronium in order to achieve muscle relaxation in studies of the human biliary excretion of rocuronium. The assay was successfully applied in a number of pharmacokinetic studies in dog and in man.

The method was used in more than 300 patients to determine the concentrations in time in various body fluids (plasma, urine, bile, stoma fluid). The plasma concentration decay of rocuronium was measured in healthy patients [9] and in patients with organ diseases, *e.g.* renal failure [10]. Also studies were performed to study the pharmacokinetics in various age groups and under different anaesthetic techniques [11]. So far, the putative metabolites, the 17-hydroxy derivative and the N-desallyl derivative could be shown neither in plasma nor in the other aforementioned body fluids. Incidental interference in the HPLC determination with other drugs, in particular with the peak associated with the N-desallyl derivative, could be successfully recognized by the additional separation using TLC.

CONCLUSIONS

Rocuronium and its putative metabolites (the 17-desacetyl derivative and the N-desallyl derivative of rocuronium) can be quantified in plasma, urine, bile, tissue homogenates and stoma fluid (rocuronium and the 17-desacetyl derivative only) by HPLC. The method is sensitive (lower limit of quantification for rocuronium in plasma is 10 ng/ml) and accurate. The interference of concomitant drugs with the assay of rocuronium and its putative metabolites has been studied extensively. In order to confirm the identity of rocuronium and its putative metabolites, a TLC method was developed. The method has been applied successfully in several pharmacokinetic studies with rocuronium.

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