Quantitative determination of rocuronium in human plasma by liquid chromatography–electrospray ionization mass spectrometry

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Abstract

Liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) was used for the quantification of the neuromuscular blocking agent rocuronium in human plasma. Verapamil was used as internal standard. The samples were subjected to a dichloromethane liquid–liquid extraction after ion pairing of the positively charged ammonium compound with iodide prior to LC–MS. Optimized conditions involved separation on a Symmetry Shield RP-18 column (50×2.1 mm, 3.5 µm) using a 15-min gradient from 10 to 90% acetonitrile in water containing 0.1% trifluoroacetic acid at 250 µl/min. Linear detector responses for standards were observed from 25 to 2000 ng/ml. The extraction recovery averaged 59% for rocuronium and 83% for the internal standard. The limit of quantification (LOQ), using 500 µl of plasma, was 25 ng/ml. Precision ranged from 1.3 to 19% (LOQ), and accuracy was between 92 and 112%. In plasma samples, at 20 and 4°C, rocuronium was stable at physiological pH for 4 h; frozen at $-30°C$ it was stable for at least 75 days. The method was found suitable for the analysis of samples collected during pharmacokinetic investigations in humans. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Neuromuscular blocking drugs (NMBDs) are widely used to promote better ventilatory management in critically ill patients with acute respiratory distress syndrome [1]. These drugs have been widely studied in anesthetized patients. Thus, most recommendations for use of NMBDs in the intensive care unit are extrapolated from short term, perioperative studies in relatively healthy patients. Consequently, more data describing the pharmacokinetic–pharmacodynamic relationships of these drugs are needed to refine its use in the intensive care unit (ICU) setting [2,3].

Among NMBDs, rocuronium given by constant infusion has proved to be useful in the management of ICU patients [4]. Rocuronium, the bromide of 1-[17β-(acetyloxy)-3α-hydroxy-2β-(4-morpholinyl)-5α-androstan-16β-yl]-1-(2-propenyl) pyrrolidinium is an aminosteroid muscle relaxant with a rapid onset

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of action and an intermediate duration of neuro-
muscular blocking effect [5,6].

Quantification of rocuronium in biological fluids
has been reported [7,8] using either high-perform-
ance liquid chromatography (HPLC) or capillary gas
chromatography (GC). With liquid chromatography
(LC) [7], the fluorescent anion 9,10-dimethoxyanth-
racene-2 sulfonate was used and a post-column ion-
pair extraction into dichloromethane with a second
pump device was implemented since rocuronium
does not present any UV absorbance. GC analysis
coupled to a nitrogen detector [8] requires deri-
vatization of rocuronium with *N*-methyl-*N*-(*tert*-
butyldimethylsilyl)-trifluoroacetamide.   Such   de-
scribed procedures are time-consuming and present
difficulties in routine operation.

The lack of UV absorbance and the presence of an
ammonium moiety prompted us to investigate
electrospray ionization mass spectrometry (ESI-MS)
to detect and identify rocuronium in human plasma.
Indeed, this soft ionization technique is particularly
well-adapted for the characterization of polar bio-
molecules [9] requiring no prior sample derivatiza-
tion and can be easily coupled to LC [10]. The
selectivity and sensitivity of LC–MS techniques
explain their extensive applications in drug develop-
ment [11]. According to previous studies [12],
verapamil was selected as internal standard. This
method was validated according to established vali-
dation procedures [13–15].

2. Experimental

2.1. Chemicals and reagents

Rocuronium was kindly supplied by Organon
Teknika (Turnhout, Belgium) and was stored in a
refrigerator (about 4°C). The internal standard (ver-
apamil) was obtained from Sigma (St. Louis, MO,
USA) and was stored with light protection at room
temperature (20°C). The structures of these com-
pounds are shown in Schemes 1 and 2. LC-grade
acetonitrile, methanol, dichloromethane and trifluoro-
acetic acid (TFA) were purchased from Merck
(Darmstadt, Germany). Sodium dihydrogenphos-
phate, orthophosphoric acid and potassium iodide
were obtained from Merck, Carlo Erba (Val de Reuil,
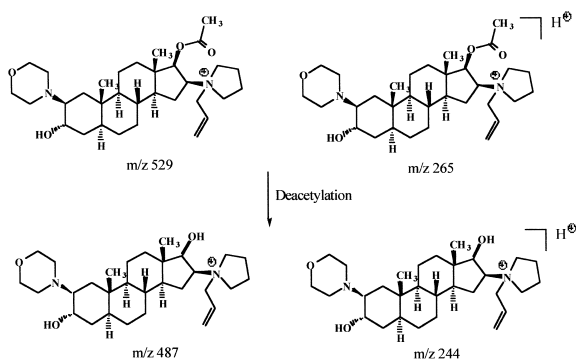
Scheme 1. Major fragmentation pathway of rocuronium.

France) and UCB (Bruxelles, Belgium), respectively;
all were of analytical grade.

To prepare the standard and the quality control
(QC) samples, pooled blank (drug-free) human plas-
ma was obtained from untreated healthy volunteers
(Etablissement Français du sang Pyrénées–Mediter-
ranée, Montpellier, France). Plasma was aliquoted,
then frozen at −30°C to be used during the study in
the preparation of standards and QCs.

Two dihydrogenphosphate buffer solutions were
prepared, 1 *M* (to use in sample pretreatment step)
and 0.1 *M* (pH 3, adjusted with orthophosphoric
acid, to prepare stock and working solutions of
rocuronium).

Saturated potassium iodide was prepared just prior
to use in distilled water by dissolving 10 g in 10 ml
(~6 *M*).

2.2. Instrumentation

ESI mass spectra were recorded on a Platform II
quadrupole mass spectrometer (Micromass, Man-
chester, UK) fitted with an electrospray ion source.
The mass spectrometer was calibrated in the positive
ion mode using a mixture of NaI and CsI. Voltages
were set at +3.5 kV for the capillary and +0.5 kV
for the skimmer lens. The source was heated at
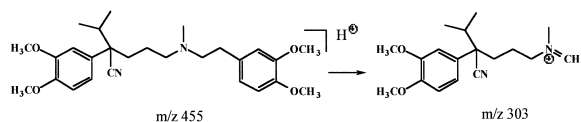
Scheme 2. Major fragmentation pathway of verapamil.

120°C. Nitrogen was used as nebulizing and drying gas at 15 and 250 l/h, respectively. The apparatus used for the LC was an Alliance 2690 (Waters, Milford, MA, USA) system equipped with an auto-sampler.

Optimization of various experimental parameters including nature of the stationary phase, composition of the eluent and sampling cone voltage were carried out. Rocuronium and verapamil samples at $5 \cdot 10^{-4}$ M were dissolved in various eluents (Table 1) and infused directly into the ESI source at a flow-rate of 35 μl/min. Flow injection analysis (FIA) mass spectrometric data were acquired in the scan mode from m/z 100 to m/z 800 in 2 s. Five scans were summed to produce the final spectrum.

LC–MS experiments were carried out by implementing a Symmetry Shield RP18 cartridge (50×2.1 mm I.D.) packed with 3.5 μm particles (Waters) between the LC pumping system set up at 250 μl/min and the split which reduced the flow-rate in the source to 35 μl/min. A linear gradient from 10 to 90% of acetonitrile in water containing 0.1% TFA was applied in 15 min. The column was then washed during 3 min at the final gradient condition, brought back to the initial condition in 1 min and equilibrated during 5 min. The total run cycle was thus equal to 24 min. Mass spectrometric data were acquired in the single ion recording (SIR) mode at m/z 455 for verapamil and m/z 265 and m/z 529 for rocuronium. The sampling cone voltage was set at 30 V.

2.3. Sample preparation procedure

The sample pre-treatment procedure involved a dichloromethane liquid–liquid extraction after ion pairing of the positively charged ammonium compound with iodide.

To 0.5 ml of plasma, 200 μl of 1 M phosphate buffer, 50 μl of internal standard (50 μg/ml), 500 μl of saturated aqueous potassium iodide solution and 5 ml of dichloromethane were added. After 30 min of extraction on a rotary mixer, the tubes were centrifuged for 15 min at 3000 g. The lower organic phase was cleaned by filtration through a phase separator silicon-treated filter paper Whatman 1 PS (Prolabo, Paris, France). The dichloromethane phase was evaporated under nitrogen stream at 30°C to dryness. The residue was dissolved in 100 μl of acetonitrile. An aliquot of 3 μl was injected onto the LC column.

2.4. Method validation

Stock solutions of rocuronium (250 μg/ml) and verapamil (internal standard, 625 μg/ml) were prepared in 0.1 M phosphate buffer (pH 3.0) and in methanol–distilled water (1:100), respectively. These solutions were stable at 4°C for at least 1 month. A solution of rocuronium and internal standard in acetonitrile (1.25 μg/ml for both compounds) was injected before each run to be analyzed to check the LC–MS system.

Calibration curves (25, 50, 100, 200, 500, 1000 and 2000 ng/ml) and QC samples (80, 400 and 1500 ng/ml), in human plasma, were used in the validation. Unweighted least-squares linear regression was used to fit the analyte/internal standard peak area ratio versus the theoretical concentration. The linearity of the method was statistically confirmed [13–15].

The precision and accuracy were determined by

Table 1
Eluents used in FIA to optimize the detection of rocuronium

Eluent number	Composition
1	CH_3CN or CH_3OH
2	CH_3CN or $CH_3OH+0.1\%$ HCO_2H
3	CH_3CN or $CH_3OH+0.1\%$ CH_3CO_2H
4	CH_3CN or $CH_3OH+0.1\%$ TFA
5	CH_3CN or CH_3OH–water (50:50)
6	CH_3CN or CH_3OH–water–HCO_2H (49.9:49.9:0.2)
7	CH_3CN or CH_3OH–water–CH_3CO_2H (49.9:49.9:0.2)
8	CH_3CN or CH_3OH–water–TFA (49.9:49.9:0.2)

analyzing, QC samples six times the same day and once a day during 6 successive days. The effect of dilution was tested from QC samples at 4000 and 8000 ng/ml. The extraction recoveries of rocuronium and internal standard from human plasma were also calculated [13–15].

2.5. Stability assays

Stability studies of rocuronium in plasma without acidification were performed, at 20 and 4°C for 6 h and at −30°C for 75 days, using QC samples at 80, 400 and 1500 ng/ml. The stability of rocuronium and verapamil in the pretreated samples under autosampler conditions was studied over a 72-h period. Stability assays were also carried out in the whole blood for 4 h at 20°C.

A drug was considered stable if more than 85% of the intact drug was retained at the end of the study period.

3. Results and discussion

3.1. Optimum conditions for LC–MS analysis

The use of slightly acidic solutions to dissolve rocuronium and verapamil favors ionization by protonation of basic sites [9]. Thus, verapamil was evidenced by the singly protonated molecule $[M+H]^+$ at m/z 455 and rocuronium by the existing quaternary ammonium salt M^+ at m/z 529 and the monoprotonated species $[M+H]^{2+}$ at m/z 265 (Scheme 1).

To improve the sensitivity of rocuronium detection, the nature of the eluent and the LC stationary phase as well as the sampling cone voltage were varied to generate a spectrum displaying a single ion.

Non acidic eluents were studied to avoid rocuronium protonation and thus to detect only M^+ whereas acidic solutions should promote extensive formation of $[M+H]^{2+}$. The influence of the nature of the eluent based on acetonitrile and methanol solutions (Table 1) was investigated by fixing the sampling cone voltage at a standard value of 30 V. The presence of small amount (0.1%) of organic acid (formic, acetic and trifluoroacetic acids) was evaluated. The relative abundances of m/z 455, 529 and
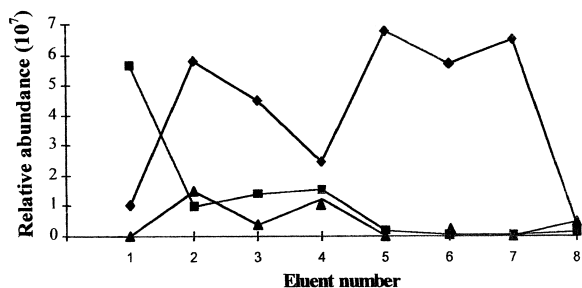

Fig. 1. Abundance of verapamil and rocuronium ions using eluents containing acetonitrile. 1: CH_3CN; 2: $CH_3CN+0.1\%$ HCO_2H; 3: $CH_3CN+0.1\%$ CH_3CO_2H; 4: $CH_3CN+0.1\%$ TFA; 5: CH_3CN–water (50:50); 6: CH_3CN–water–HCO_2H (49.9:49.9:0.2); 7: CH_3CN–water–CH_3CO_2H (49.9:49.9:0.2); 8: CH_3CN–water–TFA (49.9:49.9:0.2). The sampling cone voltage was fixed at a standard value of 30 V. m/z 455 (♦); m/z 529 (■); m/z 265 (▲).

265 ions, when using eluents containing acetonitrile, are displayed in Fig. 1. Same results were obtained when acetonitrile was replaced by methanol (data not shown). While the molecular ion of rocuronium was the most abundant in pure methanol and acetonitrile (Fig. 1, eluent 1), the $[M+H]^{2+}$ ion was also detected and all of our attempts to favor the exclusive formation of this ion by extensive protonation in acidic conditions were unsuccessful (Fig. 1, eluents 2–8). The quantitative analysis of rocuronium was thus effected by recording simultaneously the two ions at m/z 529 and m/z 265 in the SIR mode. However, the best eluents to generate abundant rocuronium ions (pure methanol or acetonitrile) were not suitable for LC analysis. Indeed, the presence of the ammonium moiety provided peak tailing on the reversed-phase column without any organic acid modifier. Thus, various acid containing mobile phases were investigated (Fig. 1, eluents 6–8). Using formic or acetic acid, rocuronium was eluted in the solvent front. Only TFA allowed good separation between rocuronium and the internal standard.

Different reversed-phase microcolumns packed with small particles were studied. Such cartridges allow to increase productivity (faster analysis and reduced solvent consumption) without sacrificing performance [16]. Fast LC is unique in its ability to effect separations at very high capacity factors while maintaining reasonable analysis time [17]. Moreover,

in hyphenated microcolumn LC–MS technology, the low flow-rate of the mobile phase enhances the sensitivity of the mass spectrometric detection. The best conditions involved chromatographic separation on a Symmetry Shield RP-18 column (50×2.1 mm) packed with particles of 3.5 μm.

Chromatographic separation being optimized, the second parameter, the sampling cone voltage, was varied to increase the abundances of rocuronium ions. Indeed, the presence of adduct and fragment ions which divide the response on several signals must be avoided to enhance the sensitivity. No adduct ions with sodium or with solvent molecules were recorded at any cone voltage. Fragmentations were only observed at high cone voltages (around 100 V) for the two compounds (Schemes 1 and 2). Thus, the cone voltage was kept at 30 V providing

the most abundant rocuronium ions with no fragmentation.

3.2. Validation results

Over the 2 months of validation, observed retention times were 6.4 (±0.12) min for rocuronium and 9.3 (±0.11) min for the internal standard. As shown in Fig. 2a, no peaks due to the matrix interfered at the retention time of the two analytes; on this chromatogram, the signal-to-noise ratio was of 7.

Representative chromatograms are shown in Fig. 2. Under the chromatographic conditions used, the number of theoretical plates was approximately 4200 for rocuronium and 7500 for verapamil; no signifi-
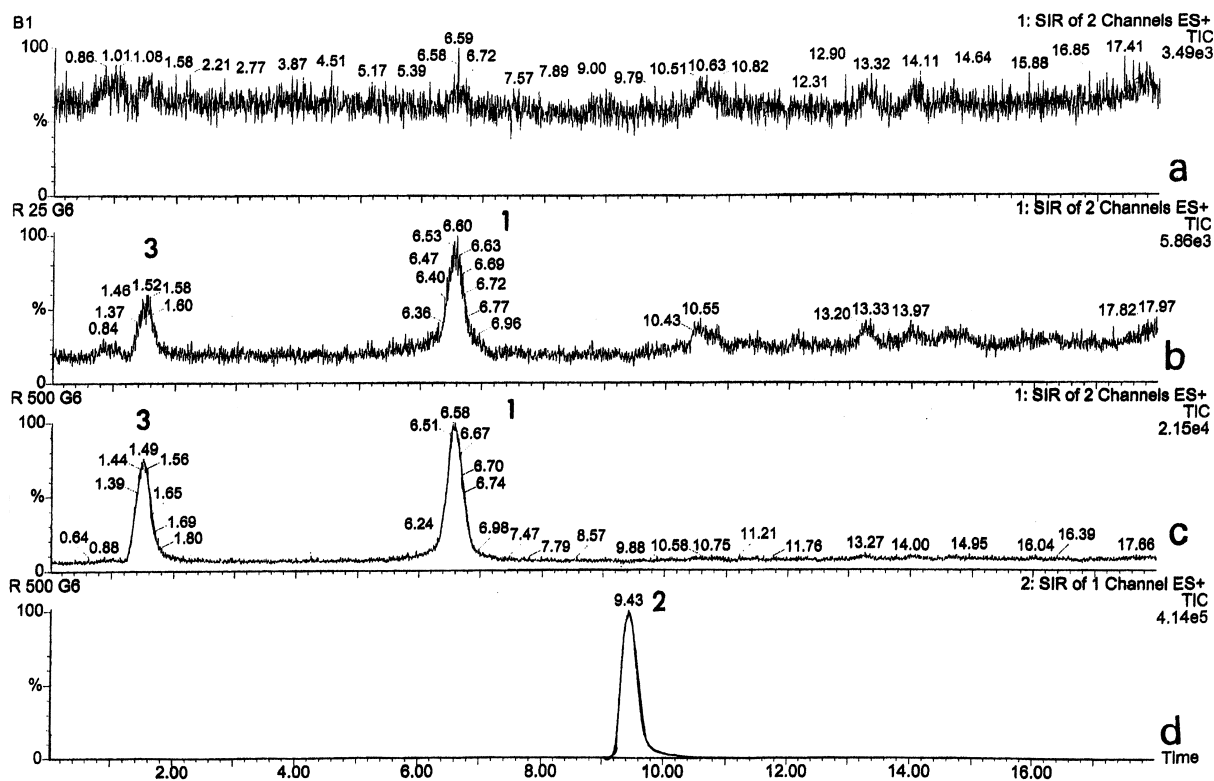

Fig. 2. Typical chromatograms of (a) blank human plasma, (b) blank plasma spiked with rocuronium at 25 ng/ml, (c) blank plasma spiked with rocuronium at 500 ng/ml, (d) blank plasma spiked with verapamil (internal standard) at 5 μg/ml. Peak 1 is rocuronium; peak 2 is verapamil; peak 3 is an additional product present in rocuronium. For LC–MS conditions see Section 2.2.

cant decrease of the number of theoretical plates occurred for the 2 months.

The assay was linear in the concentration range of 25–2000 ng/ml. The correlation coefficients (r) for calibration curves were equal to or better than 0.996. A linear regression of the back-calculated (computed from calibration curve equations) versus the nominal concentrations provided a unit slope and an intercept equal to 0 (Student's t-test). The bias (4.51) was not statistically different from zero (Student's t-test) and the 95% confidence interval included the zero value (-3.25 to 12.3). From the mean back-calculated concentrations, the recovery ranged from 92 to 112% and the relative standard deviation (RSD) did not exceed 19% ($n=84$).

Using QC samples, accuracy was between 94 and 106% and precision between 3.1 and 9.8%. The mean extraction recovery of rocuronium ($n=9$) averaged $59\pm3.5\%$ and was not statistically different over the range of concentrations studied. The extraction recovery was $83\pm6\%$ for the internal standard ($n=3$). The limit of quantification (LOQ) [13–15] was 25 ng/ml (accuracy, 94–97%; precision, 15–19%). At this level, the signal-to-noise ratio was of 33 (Fig. 2b). The dilution has no influence on the performance of the method which could then be used up to 8000 ng/ml.

3.3. Stability

In most of the published pharmacokinetic studies, plasma samples were acidified before storage to prevent degradation of rocuronium. In the present study the stability of rocuronium at physiological pH was investigated. Our results showed that rocuronium was stable in plasma samples at 20°C and 4°C for 4 h (recoveries, 99 ± 5.9 and $99\pm7.0\%$, respectively). Frozen at -30°C this drug was stable for at least 75 days. Thus, when plasma samples were frozen in the 4 h following plasma collection, their acidification was not required. Consequently, tedious manipulations could be avoided and the ruggedness of the method could be improved.

In resuspended plasma extracts in acetonitrile, rocuronium and verapamil were found to be stable for 72 h in the autosampler. Also rocuronium resists the effect of up to two freezing and thawing cycles. In blood, rocuronium was stable during 4 h.

4. Conclusion

In this study we described a LC–MS method to quantify rocuronium in human plasma. The high selectivity of MS eliminates possible interferences by concomitant drugs administered in ICU patients (lidocaine, aminosides, corticoids, cephalosporins, midazolam, flunitrazepam, ephedrine, esmolol, etc.). Indeed, a problem often met in pharmacokinetic and toxicological studies performed in such a population of patients was the interference of concomitant drugs and their potential metabolites. LC–MS conditions were optimized to obtain the best signal, stability of the measurement and the highest sensitivity. The LOQ of the method was 25 ng/ml. The sensitivity and the high selectivity of the present method as well as the validation results indicated that the performance characteristics of this method fulfilled the requirements for a sufficiently accurate and precise assay method to carry out pharmacokinetic studies.

Acknowledgements

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