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Determination of sugammadex in human plasma, urine, and dialysate using a high-performance liquid chromatography/tandem mass spectrometry assay

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ABSTRACT

Sugammadex (Bridion®, Merck Sharp & Dohme Corp., Oss, The Netherlands) is a modified γ-cyclodextrin which has the ability to reverse the neuromuscular blockade induced by the steroidal neuromuscular blocking agents rocuronium and vecuronium. The objective of the current study is to describe the bioanalytical methods that have been developed and validated according to US Food and Drug Administration guidelines on bioanalytical method validation, and subsequently applied to determine total sugammadex (i.e., free sugammadex plus sugammadex bound to the neuromuscular blocking agent) in human heparinized plasma, urine and dialysate. Sugammadex was extracted from human plasma and urine using solid phase extraction with Isolute HAX 96-well extraction plates; no extraction was performed on dialysate samples. Samples from plasma, urine, and dialysate were analyzed on a Polaris® C18-A PEEK (polyaryletheretherketone) analytical column (50 mm × 4.6 mm internal diameter, 5 μm) with a linear mobile phase gradient of 0.1% (v/v) formic acid in water:methanol from 70:30 to 20:80. The flow rate was 1 mL/min with a total run time for each injection of 6 min. Tandem mass spectrometric detection was conducted using multiple reaction monitoring under negative ion mode with a turbo ion-spray interface to quantify the concentration of sugammadex. Inter- and intra-assay precision and accuracy were within pre-defined acceptance limits. The presence of rocuronium did not interfere with the assay in plasma, urine or dialysate; similarly, vecuronium did not interfere with the plasma assay (not tested for interference in urine or dialysate). Sugammadex was found to be stable in plasma, urine and dialysate in the short-term at room temperature, in the long-term at −20 °C, and after several freeze/thaw cycles. The validated bioanalytical methods developed here have been successfully applied in a series of clinical studies for the determination of total sugammadex in plasma, urine and dialysate.

1. Introduction

Sugammadex (Bridion®, Merck Sharp & Dohme Corp., Oss, The Netherlands), per-6-(2-carboxyethylthio)-per-deoxy-γ-cyclodextrin sodium (Fig. 1a), is a modified γ-cyclodextrin developed specifically to encapsulate the steroidal neuromuscular blocking agent (NMBA) rocuronium (Fig. 1b) [1,2]. Sugammadex also encapsulates the NMBA vecuronium (Fig. 1c) with high affinity [3]. Following complex formation with rocuronium or vecuronium, sugammadex reduces the amount of NMBA available to bind to nicotinic receptors at the neuromuscular junction, resulting in rapid reversal of the neuromuscular blockade [4–7].

The use of liquid chromatography/tandem mass spectrometry (LC–MS/MS) methods to quantify total sugammadex and rocuronium in plasma and urine of guinea pigs has been described previously, with these methods successfully utilized to determine the plasma pharmacokinetics and urinary excretion of both compounds in preclinical studies [8]. In order to support pharmacokinetic studies in humans, selective and sensitive LC–MS/MS methods were developed for the determination and quantification of total sugammadex in human plasma, urine, and dialysate. Here we describe the development and validation of these methods, and their application to clinical sample analysis.

2. Materials and methods

2.1. Solvents and chemicals

Sugammadex was synthesized as previously described by Bom et al. [2] at the Merck Sharp & Dohme Corp. Laboratories, Oss, The

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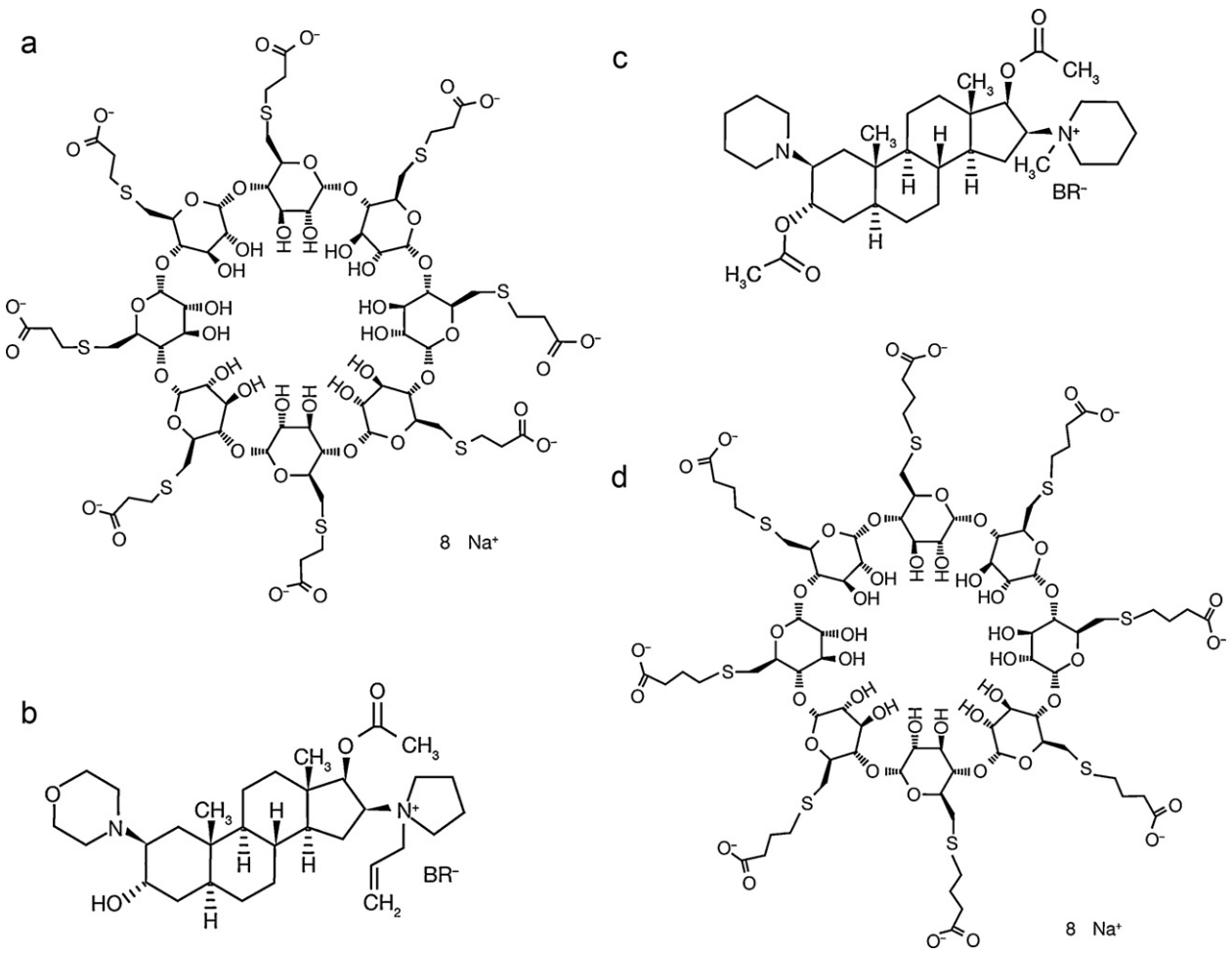

Fig. 1. Chemical structures of (a) sugammadex, (b) rocuronium, (c) vecuronium and (d) the internal standard Org 26265.

Netherlands and obtained as a 10 mg/mL (plasma and urine assays) or as a 100 mg/mL (dialysate assay) stock solution in water. The internal standard Org 26265 (per-6-(2-carboxypropylthio)-per-6-deoxy-γ-cyclodextrin sodium) was also synthesized at the Merck Sharp & Dohme Corp. Laboratories, Oss, The Netherlands (Fig. 1d). Both sugammadex and Org 26265 were supplied by the Investigational Product Supply Department of Merck Sharp & Dohme Corp., Oss, The Netherlands. Each compound was provided with a certificate of analysis containing information on identity and purity as obtained from ^{1}H- and ^{13}C-NMR, MS and HPLC measurements.

Acetonitrile (Uvasol® grade) and formic acid (99–100% Normapur grade) were obtained from Merck (Darmstadt, Germany) and VWR (Amsterdam, The Netherlands), respectively. Methanol Absolute, liquid chromatography–mass spectrometry (LC–MS) grade was obtained from Biosolve B.V. (Valkenswaard, The Netherlands). Bovine serum albumin (Fraction V, ≥98%) was obtained from Sigma–Aldrich Corporation (Saint Louis, MS, USA). Drug-free human heparinized plasma was obtained from Biological Specialty Corporation (Colmar, PA, USA). Blank urine was obtained from an internal donor pool (Merck Sharp & Dohme Corp., Oss, The Netherlands). Blank dialysate was obtained from Onze Lieve Vrouw Ziekenhuis (Aalst, Belgium). Water was purified by the Milli-Q ultra-pure water system from Millipore (Billerica, MA, USA). Isolute® 96-well solid phase extraction (SPE) plates with 25 mg Isolute® HAX sorbent material per well (Biotage AB, Uppsala, Sweden) were used for SPE of plasma and urine samples.

2.2. Standards and samples

Solutions of 1 mg/mL sugammadex were prepared in Milli-Q water by appropriate dilution of the stock solutions provided. Stock solutions of 1 mg/mL internal standard were also prepared in water by weighing the solid compound and dissolving in the appropriate volume.

2.2.1. Plasma

Each analytical run comprised the following: system suitability samples (75 ng/mL sugammadex and 375 ng/mL internal standard in methanol/0.1% (v/v) formic acid in Milli-Q water (50/50 [v/v])) at the beginning and the end; blank plasma samples (as many as needed to prevent carry over from the highest calibration and quality control [QC] samples and between study samples); blank water samples (at least one); calibration samples prepared in heparinized plasma, containing sugammadex at the following concentrations: 0.1, 0.2, 0.4, 1, 2, 4, 10, 20 and 40 μg/mL, with a fixed amount of 2 μg/mL internal standard (each measured in duplicate); QC samples containing sugammadex (range 0.1, 0.3, 4, 32, 320 and 1600 μg/mL [320 μg/mL and 1600 μg/mL samples diluted 10- and 50-fold, respectively, prior to analysis]) and 2 μg/mL internal standard in human plasma (each measured in triplicate); study samples spiked with 2 μg/mL internal standard (each measured in singular).

Samples were allowed to thaw (unassisted) at room temperature prior to further processing. If samples were <250 μL in volume, samples were adjusted to 250 μL with blank plasma. Each sample

was added to a well of a 96-well SPE plate, which had been pre-activated with 1 mL of methanol and 1 mL of 0.01% (v/v) formic acid in Milli-Q water, with a decreased vacuum applied to create a sample loading time of ≥1 min. Wells were then washed twice, first with 0.5 mL of methanol/Milli-Q water (5/95 [v/v]) and then with 0.5 mL of methanol/Milli-Q water (75/25 [v/v]). Following this, an increased vacuum was used to aspirate the sorbent material to dryness before elution of the samples with 0.5 mL of 5% (v/v) formic acid in methanol using a decreased vacuum. The eluates were collected in the wells of a masterblock. The elution step was then repeated and the eluate collected in the same masterblock. The eluates were evaporated to dryness using a 96-well sample concentrator at ∼60 °C, under a gentle stream of nitrogen. Residues were dissolved in the masterblock in methanol/0.1% (v/v) formic acid in Milli-Q water (50/50 [v/v]) and the masterblock was sealed using polypropylene-coated aluminium foil. Samples were homogenized using a vortexer and subsequently analyzed using LC–MS/MS (see Section 2.3).

Analytical runs were accepted if the determination coefficient (*r*-squared) was ≥0.98, the accuracy of at least two-thirds of all calibration samples was between ±20% (including limits) with at least one calibration sample of the highest and the lowest concentrations accepted accordingly, and the accuracy of at least two out of three of the lowest QC samples was between ±20% (including limits) and the accuracy of at least two out of three of the other QC samples was between ±15% (including limits).

2.2.2. Urine

Each analytical run comprised: system suitability samples (2.5 μg/mL sugammadex and 2.5 μg/mL internal standard in Milli-Q water) at the beginning and the end; blank urine samples (typically two blanks were sufficient to remove carry over, but were repeated as necessary); blank water samples (at least one); calibration samples prepared from pooled blank urine containing sugammadex at concentrations of 5, 10, 20, 50, 100, 150 and 200 μg/mL, together with a fixed amount of 20 μg/mL internal standard (each measured in duplicate); QC samples (5, 15, 80, 150 and 1500 μg/mL sugammadex [1500 μg/mL sample diluted 10-fold prior to analysis] plus 20 μg/mL internal standard prepared in human urine) (each measured in triplicate); and study samples spiked with 20 μg/mL internal standard (each measured in singular). An equal volume of blank human plasma was added within 1 h after sample collection at the clinical site to each study sample to stabilize the sugammadex prior to freezing; similarly, each blank, calibration and QC sample also had blank human plasma added in a 1:1 ratio.

Samples were allowed to thaw (unassisted) at room temperature prior to further processing. If sample volumes were less than 200 μL, samples were adjusted to 200 μL with blank urine/plasma mix. Samples were added to the wells of a 96-well SPE plate, which had been pre-activated with 1 mL of methanol and 1 mL of 0.01% (v/v) formic acid in Milli-Q water, with a sample loading time of ≥1 min by vacuum. Wells were then washed twice, first with 0.5 mL of methanol/Milli-Q water (5/95 [v/v]) and then with 0.5 mL of methanol/Milli-Q water (75/25 [v/v]), before applying an increased vacuum. Elution was performed with 1 mL of 20% (v/v) formic acid in ethylacetate and eluates were collected in the masterblock and evaporated to dryness using a 96-well sample concentrator at ∼60 °C, under a gentle stream of nitrogen. The resulting residues were dissolved in 400 μL of methanol/0.1% (v/v) formic acid in Milli-Q water (50/50 [v/v]), then the masterblock was sealed using polypropylene-coated aluminium foil and the samples were homogenized using a vortexer. Samples were analyzed by LC–MS/MS (see Section 2.3).

Analytical runs were accepted provided the accuracy of at least two-thirds of all calibration samples was ±15% (including limits), or ±20% (including limits) for the lower limit of quantification (LLOQ), with at least one calibration sample of the highest and the lowest concentrations accepted accordingly. Further, the accuracy of at least one QC sample for each concentration level needed to be ±15% (including limits), with at least one QC sample at the beginning and end of the analytical run, as well as two-thirds of all QC samples required to be accepted accordingly.

2.2.3. Dialysate

Standard calibration samples were freshly prepared in human dialysate in duplicate before each analytical run at the following concentrations: 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50 μg/mL sugammadex. QC samples were similarly prepared (0.1, 0.3, 2, 40 and 4000 μg/mL [4000 μg/mL sample diluted 100-fold prior to analysis]). Internal standard was added to a concentration of 15 μg/mL to each calibration, QC, and study sample.

A system suitability test (15 ng/mL sugammadex and 2 μg/mL internal standard in Milli-Q water) was performed at the beginning and end of each run. Each analytical run also comprised at least one blank dialysate sample (depending on the concentration of the samples being tested further blanks could be included to sufficiently prevent carry over), at least one blank water sample, duplicate calibration samples, duplicate QC samples, and study samples.

Dialysate samples were allowed to thaw (unassisted) at room temperature, added to a 96-well collection plate and mixed for approximately three seconds using a whirlmixer. Where volumes less than 100 μL were available, samples were adjusted to 100 μL with blank dialysate. After addition of internal standard, 600 μL of Milli-Q water was then added to all samples. The masterblock was then sealed with polypropylene-coated aluminium foil and the samples were homogenized using a vortexer. Samples were then analyzed by LC–MS/MS (see Section 2.3). No extraction was performed on dialysate samples.

Analytical runs were accepted provided the accuracy of ≥75% of all calibration samples was between ±15% (including limits), or ±20% (including limits) for the LLOQ, with at least one of the lowest and the highest concentrations of the calibration samples accepted accordingly. The accuracy of ≥50% of the QC samples at each concentration level was also required to be ±15% (including limits), or ±20% (including limits) for the LLOQ, and at least one QC sample at the beginning of the analytical run, one at the end, and at least two-thirds of all QC samples needed to be accepted accordingly.

2.3. Instrumentation

Chromatographic separations were carried out using an HP1100 series Liquid Chromatograph (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump and a degasser. The sample injection volume of 10 μL was injected by a HTS PAL autosampler (CTC Analytics, Switzerland) and pumped at a flow-rate of 1.0 mL/min through a Polaris C_{18}-A PEEK (polyaryletheretherketone) analytical column (50 mm length × 4.6 mm internal diameter, 5 μm particle size; Varian, Inc., Palo Alto, CA, USA) with a linear mobile phase gradient of 0.1% (v/v) formic acid in water (solvent A) and methanol (solvent B) at ratios of 70:30 to 20:80. The run time was 6 min. The autosampler injection syringe was extensively washed (6–8 wash cycles) with acetontrile/methanol/0.1% (v/v) formic acid in Milli-Q water 40:40:20 (v/v) after each injection, to minimize carry-over.

The liquid chromatography eluate was split 1:10 before entering a Perkin-Elmer Sciex API 3000 triple-quadrupole mass spectrometer (Sciex Thornhill, ON, Canada) equipped with a turbo ion-spray interface (Applied Biosystems, Foster City, CA, USA). The quadrupoles were operated with unit resolution in the negative ion mode. The resulting multiple reaction monitoring chromatograms were integrated using Analyst version 1.2 or 1.4.1 software (Sciex).

Watson® Drug Metabolism Laboratory Information Management System (version 6.3.0.03 or 7.2.0) software (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) was used for quantification.

The ESI-MS analysis was performed in the negative ion mode. The quantification of sugammadex was measured using collision-induced dissociation tandem mass spectrometry (CID-MS/MS) by means of the multiple reaction monitoring (MRM) scans of the mass transitions of sugammadex of the deprotonated doubly charged molecule ($C_{72}H_{104}O_{48}S_8{\cdot}Na_8$) $[M-2H]^{-2}$ at m/z 1000.0 → m/z 963.5 and the internal standard ($C_{80}H_{120}O_{48}S_8{\cdot}Na_8$) deprotonated doubly charged molecule $[M-2H]^{-2}$ at m/z 1055.5 → m/z 119.0. Dwell time was optimized at 500 ms, Turbo Ion Spray Heater Gas was set to 8 L/min, ionspray voltage was kept at −4500 V, with a source temperature of 300 °C. Collision-induced dissociation was performed using N_2 as the collision gas with a pressure of 3.7×10^{-5} Torr. Collision cell energy was −48 eV for sugammadex and −110 eV for the internal standard.

2.4. Calibration and calculations

Calibration curves were constructed for sugammadex using linear weighted regression (weighing factor = $1/Y^2$, where Y = response ratio [test substance/internal standard]), which was found to be the optimal regression method. Mass chromatograms of the samples were processed and the data analyzed accordingly against the constructed calibration curves to calculate sugammadex levels in each sample.

2.5. Validation

2.5.1. Linearity

The linearity of each assay was evaluated by replicate analysis of a number of calibration standards (plasma: nine standards, ranging from 0.1–40 μg/mL sugammadex, with 2 μg/mL internal standard per sample; urine: seven standards, ranging from 5–200 μg/mL sugammadex, with 20 μg/mL internal standard per sample; dialysate: nine standards, ranging from 0.1–50 μg/mL sugammadex, with 15 μg/mL internal standard per sample).

2.5.2. Sensitivity

The LLOQ was defined as the lowest concentration of the calibration curve that could be determined with an inter- and intra-run accuracy of ±20% and an inter- and intra-run precision of ≤20% assessed in three analytical runs. The upper limit of quantification (ULOQ) was defined as the highest concentration of the calibration curve that could be determined with an inter-run accuracy of ±15% and an inter-run precision of ≤15% assessed over three analytical runs.

2.5.3. Selectivity

To determine possible interference from endogenous and exogenous substances in plasma, blank human plasma samples from 10 different donors were analyzed, along with blank water. These blank plasma samples were also spiked with 0.1 μg/mL sugammadex and 2 μg/mL internal standard and subsequently analyzed and compared with blank human plasma samples. The response of interfering peaks in the blank samples at the retention time of sugammadex and internal standard should be less than 20% and 5%, respectively, of the response of both compounds obtained from the spiked samples. Similarly, potential interference was also analyzed in triplicate using blank urine samples from six different donors (spiked with 5 μg/mL sugammadex and 20 μg/mL internal standard) and dialysate samples from two different donors (spiked with 0.1 μg/mL sugammadex and 15 μg/mL internal standard), compared with unspiked urine or dialysate blanks from the same donors.

The influence of rocuronium, which is likely to be present in samples taken from surgical patients, was also investigated by spiking sugammadex QC samples of plasma, urine, and dialysate with a range of rocuronium concentrations, up to the anticipated maximum concentrations in clinical samples of 40, 200 and 20 μg/mL rocuronium, respectively. The influence of vecuronium and pancuronium was investigated only in plasma samples with up to 8 μg/mL NMBA. The mean relative bias of the spiked plasma QC samples should be within ±20% for the low QC and within ±15% of all other QC samples; for urine and dialysate, the mean accuracy of the QC samples should be within ±15%.

2.5.4. Accuracy and precision

Assay accuracy and precision were determined by multiple analyses of numerous analytical runs of QC samples (range 0.1–32 μg/mL sugammadex in human plasma [320 μg/mL and 1600 μg/mL samples diluted 10- and 50-fold, respectively, prior to analysis], 5–150 μg/mL sugammadex in urine [1500 μg/mL sample diluted 10-fold prior to analysis], and 0.1–40 μg/mL sugammadex in dialysate [4000 μg/mL sample diluted 100-fold prior to analysis]).

Accuracy was expressed as a percentage deviation from the nominal concentration, and precision was calculated as the coefficient of variation (CV) according to the following equations:

$$\text{accuracy (\% deviation)} = \frac{X - C}{C} \times 100\%$$

$$\text{precision (CV\%)} = \frac{\text{standard deviation}}{X} \times 100\%$$

where X = mean calculated sugammadex concentration; C = nominal concentration; % deviation = percentage deviation of nominal concentration.

2.5.5. Recovery

Recovery of sugammadex following extraction from plasma was assessed by comparison of extracted samples with standard solutions of sugammadex in solvent, containing 0.3, 4, and 32 μg/mL sugammadex and 2 μg/mL internal standard. These standard solutions were analyzed by sixfold analysis directly on the LC–MS system to obtain maximum values. Recovery of sugammadex and the internal standard from plasma samples was then calculated by comparing peak area ratios obtained from LC–MS analysis of the extracted samples with the peak area ratios of the standard solutions containing the same concentrations of sugammadex and internal standard.

Recovery of sugammadex from urine was assessed in triplicate at 15, 80 and 150 μg/mL. Blank urine samples were spiked with each of these concentrations of sugammadex, together with 20 μg/mL internal standard. For each concentration, the signal was compared to the signal obtained by spiking the blank matrix at the same concentrations in triplicate after SPE but before evaporation and LC–MS/MS.

Recovery was not assessed from dialysate as samples were diluted (no extraction performed).

2.5.6. Matrix effect

For both plasma and urine, the ion-suppression of sugammadex and the internal standard was assessed by the addition of sugammadex and internal standard at the concentration of the low quality control sample to the final solution of each of six blank human plasma and urine samples after complete sample preparation. For reference purposes, solutions in injection solvent were prepared containing sugammadex and internal standard at the same concentrations, i.e. 0.3 μg/mL sugammadex and 2 μg/mL internal standard in the case of plasma, and 15 μg/mL sugammadex and 20 μg/mL

internal standard in the case of urine. The ion-suppression effect for both sugammadex and internal standard was assessed by comparing the mean response of each analyte in the extracted samples with the mean response of each analyte in standard solutions at the same concentration determined by sixfold injection, and calculated as matrix effect (%) = 100 − (mean peak area in presence of matrix/mean peak area in the absence of matrix) × 100. The internal standard normalized matrix factor was calculated from the peak area ratios of sugammadex and internal standard according to following formula:

$$MF_{IS\ Normalized} = \frac{\text{peak area ratio from extracted samples}}{\text{mean peak area ratio from reference solutions}}$$

where the peak area ratio is the peak area of sugammadex divided by the peak area of internal standard. The coefficient of variation (precision CV%) of the internal standard normalized matrix factor was not allowed to exceed 15%.

For dialysate, the matrix effect was evaluated using a different method as only a limited number of different matrix lots (two) were available. The matrix effect of sugammadex was therefore assessed by multiple injections of diluted blank human dialysate samples (from two different donors) during continuous post column infusion of a sugammadex solution producing a background signal comparable to the maximum peak intensity obtained for QC LLOQ. For the analysis of each sample, the LC–MS conditions were applied during at least twice the regular run time.

2.5.7. Freeze/thaw analysis

To determine the influence of repeated freezing and thawing, QC samples were stored at −20 °C and then allowed to thaw (unassisted) at room temperature. This process was repeated a number of times before analysis against a reference sample (one freeze/thaw cycle). For plasma and urine the mean results of QC samples at each concentration level after a number of freeze/thaw cycles were required to be within ±15% of the mean value of the reference samples for a valid result, whereas for dialysate the mean results of QC samples at each concentration level after a number of freeze/thaw cycles were required to be within ±15% of the nominal value.

2.5.8. Stability

Long-term stability of sugammadex in plasma, urine and dialysate samples was determined following storage of stability QC samples at −20 °C. To demonstrate long-term stability, stability QC samples were required to have a mean value within pre-determined limits of the nominal value upon re-analysis (typically ±15% or ±20% of the nominal value depending on the assay). The short-term stability of sugammadex in plasma, urine, and dialysate at room temperature was also investigated by comparison of stability QC samples stored at room temperature for at least 24 h for plasma and dialysate samples, and at least 72 h for urine samples, compared to freshly processed QC samples which were frozen directly after preparation; the mean results of those stability QC samples stored at room temperature were not allowed to differ by more than ±15% from the mean results from freshly processed QC samples.

3. Results and discussion

3.1. Method development and optimization

3.1.1. Mass spectrometry

Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources were evaluated in both the positive and negative modes. Molecular ions of sugammadex and its internal standard were determined using direct and post column infusion of an aqueous solution into the mobile phase flow. It was found that the most intense signals were obtained when negative electrospray ionization (with a turbo ion-spray interface) was applied.

The strongest molecular ions observed in the full scan mass spectrum were the double charged precursor ions: for sugammadex m/z 1000.0 and for the internal standard m/z 1055.5 as shown in Fig. 2a and b. Source temperature, ion spray voltage and gas flow settings were optimized for the highest signal (see Section 2.3). Product ions of sugammadex and the internal standard were determined by fragmenting the corresponding precursor ions using different collision energy (CE) values. As can be seen from Fig. 2c and d, the most abundant ions in the product ion mass spectra were identified at m/z 963.5 for sugammadex and at m/z 119.0 for the internal standard. These product ions were selected to obtain maximum sensitivity. The different mass transitions and corresponding CE values are reported in Section 2.3.

3.1.2. Liquid chromatography

Initial chromatography was obtained on a Hypersil BDS C18, polar endcapped reversed phase column (Thermo Fisher Scientific Inc., Waltham, MA, USA). Due to the adsorption of sugammadex to metal surfaces and observed excessive peak tailing, chromatographic separation was subsequently achieved on a Varian Polaris C18, hydrophilic groups embedded, reversed phase column (Varian Inc. Lake Forest, CA, USA). Both column types are often applied to retain polar analytes and are stable over a wide pH range (1.5–10) and under hydrophilic conditions (from zero to 100% aqueous). Sugammadex and its internal standard are both very polar compounds due to the polycarboxylic groups present. Various combinations of methanol or acetonitrile and 0.1% (v/v) formic acid in water were investigated and compared to identify the optimal mobile phase composition that produced the best sensitivity, efficiency, and peak shape. A linear mobile phase gradient was selected, starting at 30% methanol in 0.1% (v/v) formic acid (in water) and raising methanol content from 30 to 80% in 3.5 min. It was also important for the samples to avoid metal surfaces during liquid chromatography; to prevent adsorption and excessive peak tailing, PEEK-lined or full-PEEK columns were used for the assay, with all tubing also being PEEK. Each chromatographic run was completed within 6 min.

3.1.3. Sample preparation

For sample clean-up and analyte extraction, a wide range of reversed phase and mixed mode solid-phase extraction materials, combining non-polar and strong anion exchange interactions, were explored. NEXUS (Varian), a high surface area polymer with non polar retention properties, Oasis MAX (Waters S.A.S, Saint-Quentin En Yvelines, France), a mixed mode anion exchange and reversed-phase sorbent for acids, Oasis HLB (Waters), a hydrophilic–lipophilic balance reversed-phase sorbent, Bond Elute C18 (Varian) hydrophobic bonded silica sorbent, and Isolute HAX (Biotage), a mixed mode strong anion exchange hydrophobic sorbent for extraction of acids, were all investigated. Isolute HAX was selected as this type of material is especially suitable for the extraction of acidic drugs from biological fluids and was found to give the highest and most consistent recoveries. Although *in vitro* sugammadex binding studies have shown that sugammadex does not bind to human plasma proteins [12], extraction recovery of sugammadex from plasma and urine was rather low, ranging from 29 to 40% for plasma and from 44 to 46% for urine (to which an equal volume of plasma was added). It was found that sample loading onto the solid phase cartridge needed to be performed slowly to sufficiently retain sugammadex upon loading the solid phase cartridge and to obtain consistent recoveries upon elution. For urine, this could only be achieved after the addition of an equal amount of plasma. In this way, recovery of sugammadex from both

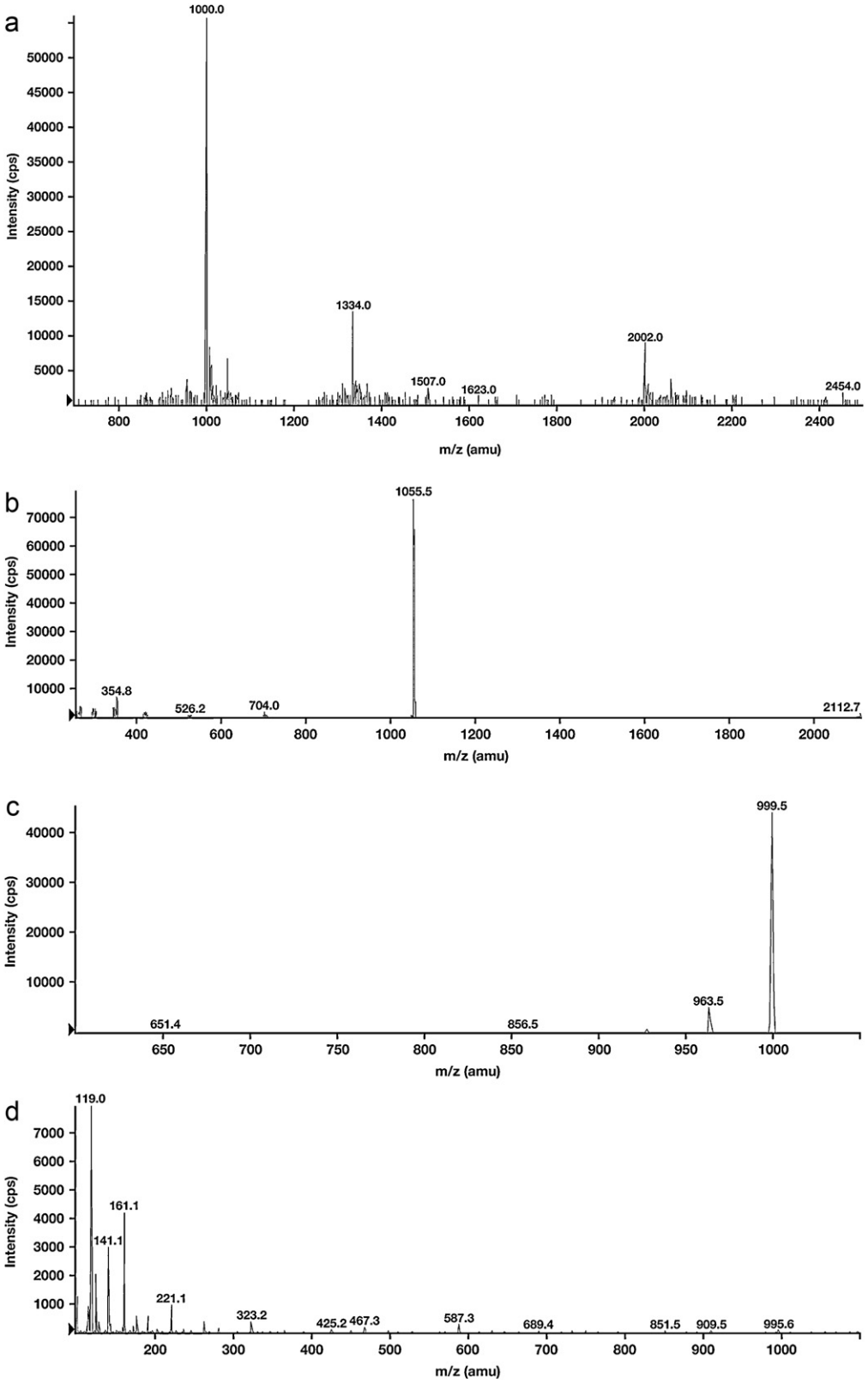

Fig. 2. Representative parent ion mass spectra of (a) sugammadex and (b) internal standard, and product ion spectra of (c) sugammadex and (d) internal standard.

plasma and urine was considered to be consistently reproducible and sufficiently high enough to analyze clinical samples in the range required with acceptable accuracy and precision. Reproducibility of recovery is particularly important when an analogue internal standard is used.

3.2. Method validation

3.2.1. Plasma

The recoveries of sugammadex following solid phase extraction ranged from 29 to 40% at 0.3, 4 and 32 μg/mL, while the recov-

Table 1
Inter-assay precision and accuracy for sugammadex in human plasma, urine, and dialysate quality control samples.

	Concentration, μg/mL	*n*	Mean	SD	Accuracy, % deviation	Precision, CV %
Plasma	0.1	21	0.106	0.007	6.0	6.4
	0.3	20	0.299	0.017	−0.2	5.5
	4	21	4.16	0.21	4.0	4.9
	32	20	31.2	2.1	−2.6	6.7
	320[a]	21	331	24	3.4	7.3
	1600[b]	–	–	–	–	–
Urine	5	17	5.07	0.190	1.4	3.7
	15	18	15.3	0.864	2.0	5.6
	80	18	81.5	2.44	1.9	3.0
	150	18	151	7.05	0.7	4.7
	1500[c]	17	1460	57.8	−2.7	4.0
Dialysate	0.1	9	0.104	0.007	4.0	6.7
	0.3	9	0.304	0.012	1.3	4.0
	2	9	2.06	0.018	3.0	0.9
	40	9	38.1	0.52	−4.8	1.4
	4000[d]	9	3850	81.9	−3.8	2.1

[a] Diluted 10-fold prior to analysis with blank plasma.
[b] Diluted 50-fold prior to analysis with blank plasma; no inter-run data established.
[c] Diluted 10-fold prior to analysis with blank urine.
[d] Diluted 100-fold prior to analysis with blank dialysate.

ery of the internal standard was 36% at 2 μg/mL. The LLOQ for the assay was 0.1 μg/mL while the ULOQ was 40 μg/mL. Inter- and intra-assay precision and accuracy are presented in Tables 1 and 2, respectively. Inter-assay precisions were within 7.3%, while accuracies were within 6.0% (expressed as % deviation from nominal). Intra-assay precision and accuracy were within 5.6% and 4.3% (expressed as % deviation from nominal), respectively. Fig. 3a and b show example chromatograms for quality control samples containing sugammadex at the LLOQ for the assay, 0.1 μg/mL, and internal standard 2 μg/mL, and sugammadex 4 μg/mL and internal standard 2 μg/mL, respectively. Retention times were 3.58–3.60 min for sugammadex and 4.11–4.13 min for internal standard.

The freeze–thaw stability of sugammadex in plasma was investigated after four freeze–thaw cycles and found to be stable, with the difference between one freeze–thaw cycle and four freeze–thaw cycles varying by <10% over the 0.3–32 μg/mL QC sample range, as well as with the 10-fold dilution of the 320 μg/mL QC sample. Similarly, neither long-term storage at −20 °C (stored over a period of 1 year and 11 months) nor storage at room temperature (over 24 h) affected the plasma sugammadex concentrations, indicating stability under each of these respective conditions.

Blank human plasma samples from 10 different donors, as well as blank water, did not exhibit a significant response at the retention times of sugammadex and Org 26265, demonstrating that there was no significant interference from endogenous and/or exogenous compounds. Furthermore, samples spiked with up to 40 μg/mL rocuronium or up to 8 μg/mL vecuronium were within the pre-defined accuracy limits, demonstrating that the presence of rocuronium or vecuronium does not influence the assay results for sugammadex. Similarly, the related aminosteroidal NMBA pancuronium 8 μg/mL did not interfere with the assay results.

The ion-suppression from human plasma for sugammadex and internal standard is shown in Table 3. The ion-suppression from human plasma was 14.3% for sugammadex at 0.3 μg/mL and 6.1% for the internal standard at 2 μg/mL. The coefficient of variation of the internal standard normalized matrix factor for human plasma was 11.7%, within the set criterion of 15%.

Sugammadex was found to exhibit significant carry-over in this assay, and therefore, during the validation, blank extracted plasma samples were injected after the highest calibration sam-

Table 2
Intra-assay precision and accuracy for sugammadex in human plasma, urine, and dialysate quality control samples.

	Concentration, μg/mL	*n*	Mean	SD	Accuracy, % deviation	Precision, CV %
Plasma	0.1	6	0.104	0.005	4.3	5.1
	0.3	5	0.291	0.015	−3.1	5.1
	4	6	4.10	0.14	2.4	3.4
	32	5	31.1	1.7	−2.9	5.6
	320[a]	6	333	17	4.0	5.1
	1600[b]	3	1550	121	−3.1	7.8
Urine	5	5	5.00	0.254	0.0	5.1
	15	6	14.7	0.341	−2.0	2.3
	80	6	82.1	2.37	2.6	2.9
	150	6	151	3.06	0.7	2.0
	1500[c]	5	1440	23	−4.0	1.6
Dialysate	0.1	5	0.104	0.003	4.0	2.9
	0.3	5	0.298	0.010	−0.7	3.3
	2	5	2.06	0.013	3.0	0.7
	40	5	38.4	0.43	−4.0	1.1
	4000[d]	5	3870	102	−3.3	2.6

[a] Diluted 10-fold prior to analysis with blank plasma.
[b] Diluted 50-fold prior to analysis with blank plasma.
[c] Diluted 10-fold prior to analysis with blank urine.
[d] Diluted 100-fold prior to analysis with blank dialysate.

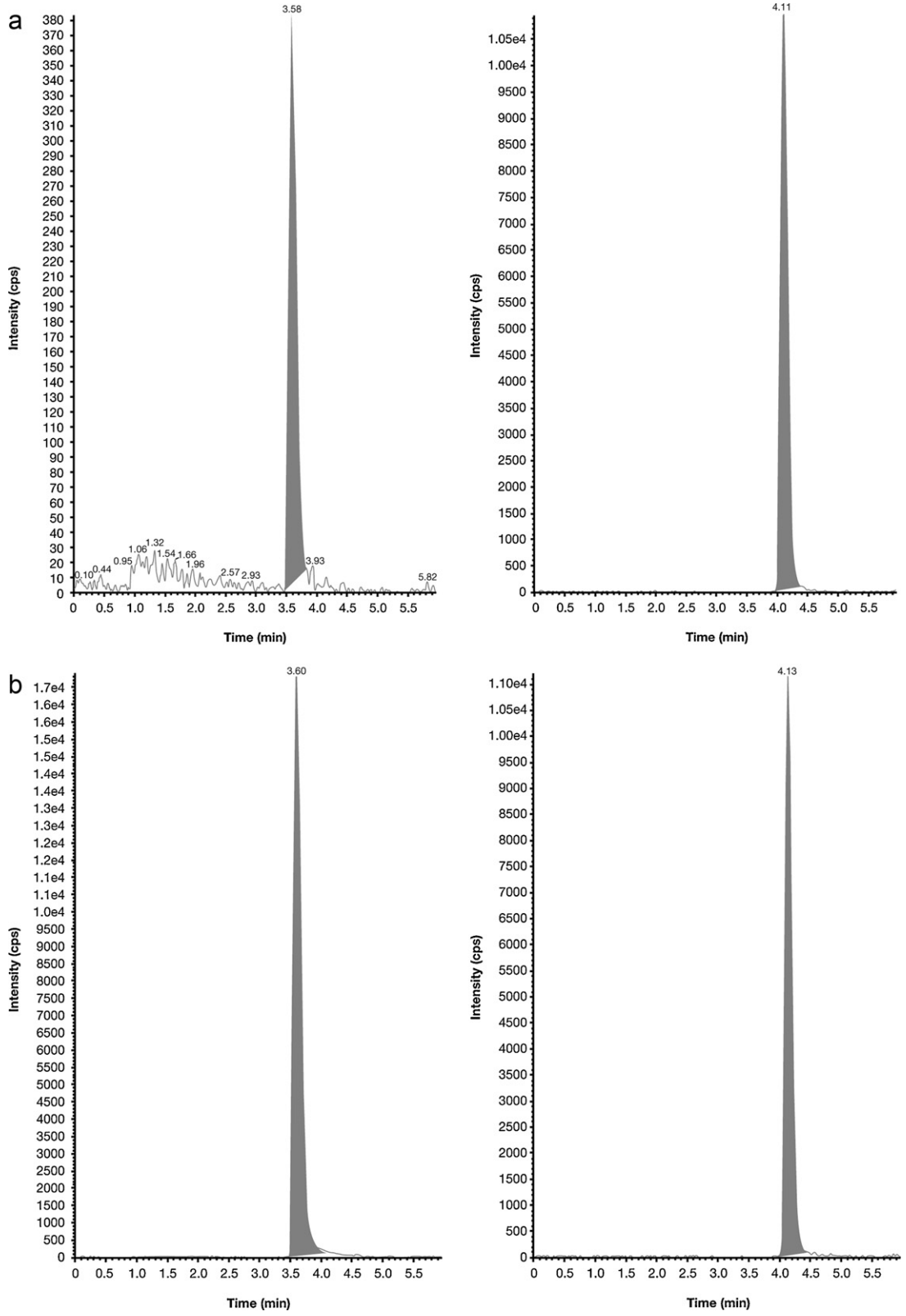

Fig. 3. Example chromatograms of sugammadex at (a) the lower limit of quantification, 0.1 μg/mL and (b) 4 μg/mL, both with the internal standard Org 26265 2 μg/mL in human plasma extract. In each case, the chromatograms on the left show sugammadex, while the chromatograms on the right show the internal standard.

ples and after the highest QC samples. During the application of this method to clinical study samples, it was important to minimize any potential carry-over; therefore, when analyzing clinical samples, typically four blank extracted plasma samples were injected after the highest calibration sample and after the highest QC-sample to monitor the occurrence of carry-over. If carry-over was detected, the impact on sample analysis was evaluated and if necessary re-analysis was performed.

Table 3
Ion suppression from human plasma.

	[Sugammadex] 0.3 μg/mL	[Internal standard] 2 μg/mL	Peak area ratio	Matrix factor (IS normalized)
	Peak area	Peak area		
Extracted samples				
	7829	192,585	0.0407	0.866
	7830	195,502	0.0401	0.853
	10,771	215,553	0.0500	1.065
	7773	195,798	0.0397	0.846
	6048	162,333	0.0373	0.794
	10,268	217,466	0.0472	1.006
Mean	8420	196,540	0.0425	0.905
%CV				11.7
Reference solutions				
	9909	207,501	0.0478	
	9965	206,602	0.0482	
	10,056	209,359	0.0480	
	9523	208,788	0.0456	
	9575	212,250	0.0451	
	9899	211,181	0.0469	
Mean	9821	209,280	0.0469	
Matrix effect (ion suppression)	14.3%	6.1%		

3.2.2. Urine

Recoveries of sugammadex following solid phase extraction were 44.7, 43.9 and 46.1% at 15, 80 and 150 μg/mL, respectively; the recovery of the internal standard was 56.3% at 20 μg/mL. The LLOQ for sugammadex in human urine was 5 μg/mL and the ULOQ was 200 μg/mL. Inter- and intra-assay precision and accuracy are presented in Tables 1 and 2; respectively. Inter-assay precisions were within 5.6%, while accuracies were within 2.7% (expressed as % deviation from nominal). Intra-assay precision and accuracy were within 5.1% and 4.0% (expressed as % deviation from nominal), respectively. Fig. 4a and b show example chromatograms for quality control samples containing sugammadex 5 μg/mL (LLOQ), and internal standard 20 μg/mL; and sugammadex 80 μg/mL and internal standard 20 μg/mL, respectively. Retention times in urine were 3.40–3.47 min for sugammadex and 3.90–3.93 for internal standard.

Freeze/thaw stability was determined over four freeze/thaw cycles, with the mean results of four freeze/thaw cycles within the pre-defined limits of ±15% compared with the mean of one freeze/thaw cycle. Sugammadex was stable in urine samples containing equal volumes of plasma when stored at room temperature for 72 h and for 1 h when stored at ∼37 °C. Sugammadex (15–150 μg/mL) concentrations were also stable when stored for 37 weeks at −20 °C.

Similar to plasma samples, the assay results for sugammadex in urine samples were not affected by the presence of rocuronium (samples spiked at a concentration of up to 200 μg/mL rocuronium). Moreover, there were no interfering peaks from endogenous substances, assessed using donated urine from six healthy individuals.

The ion-suppression from human urine for sugammadex and internal standard is shown in Table 4. The ion-suppression from human urine was 31.0% for sugammadex at 15 μg/mL and 22.9% for the internal standard at 20 μg/mL. The coefficient of variation of the internal standard normalized matrix factor for human urine was 2.5%, within the set criterion of 15%.

During LC–MS analysis of extracts from urine samples, significant carry-over was observed. This was resolved by injecting blank extracted human urine samples after the highest calibration standard, the highest quality control sample, and study samples (if required), before starting the next sample analysis with an expected concentration at or below LLOQ. When monitoring indicated the presence of carry-over, the impact on sample analysis was evaluated and if necessary re-analysis was performed. Typically two blanks appeared to be sufficient to minimize the impact of carry-over.

3.2.3. Dialysate

The LLOQ was established as 0.1 μg/mL in dialysate samples, while the ULOQ was found to be 50 μg/mL. Fig. 5a and b show representative LC–MS/MS chromatograms for quality control samples containing sugammadex 0.1 μg/mL (LLOQ) and internal standard 15 μg/mL; and sugammadex 2 μg/mL and internal standard 15 μg/mL. Retention times in these assays were 3.44–3.45 min for sugammadex and 4.00 min for the internal standard.

Inter- and intra-assay precision and accuracy are presented in Tables 1 and 2; respectively. Inter-assay precisions were within 6.7%, while accuracies were within 4.8% (expressed as % deviation from nominal). Intra-assay precision and accuracy were within 3.3% and 4.0% (expressed as % deviation from nominal), respectively.

Repeated freezing and thawing had no effect upon the concentration of sugammadex in human dialysate, tested over four freeze/thaw cycles. Sugammadex was stable in dialysate at room temperature for at least 24 h. The long-term stability of sugammadex in human dialysate, stored at −20 °C, was demonstrated for 47 weeks. No interference was detected in the assay from either endogenous or exogenous substances, and there was no interference from rocuronium in QC samples spiked with 20 μg/mL rocuronium, as the mean accuracy of the spiked samples was within the predefined limits of ±15%.

For dialysate, no significant matrix effect was observed (Fig. 6a and b).

Carry-over was assessed in one analytical run by injection of 10 processed blank human dialysate samples after each highest calibration and highest QC standard. The injection of at least four blank human dialysate samples was found to be required before starting the next sample analysis with an expected concentration at or below the LLOQ. When monitoring indicated the presence of carry-over, the impact on sample analysis was evaluated and if necessary re-analysis was performed.

3.3. Assay application to clinical samples

The validated method to determine total sugammadex concentrations in plasma after intravenous dosing has now been successfully applied in numerous clinical studies of sugammadex.

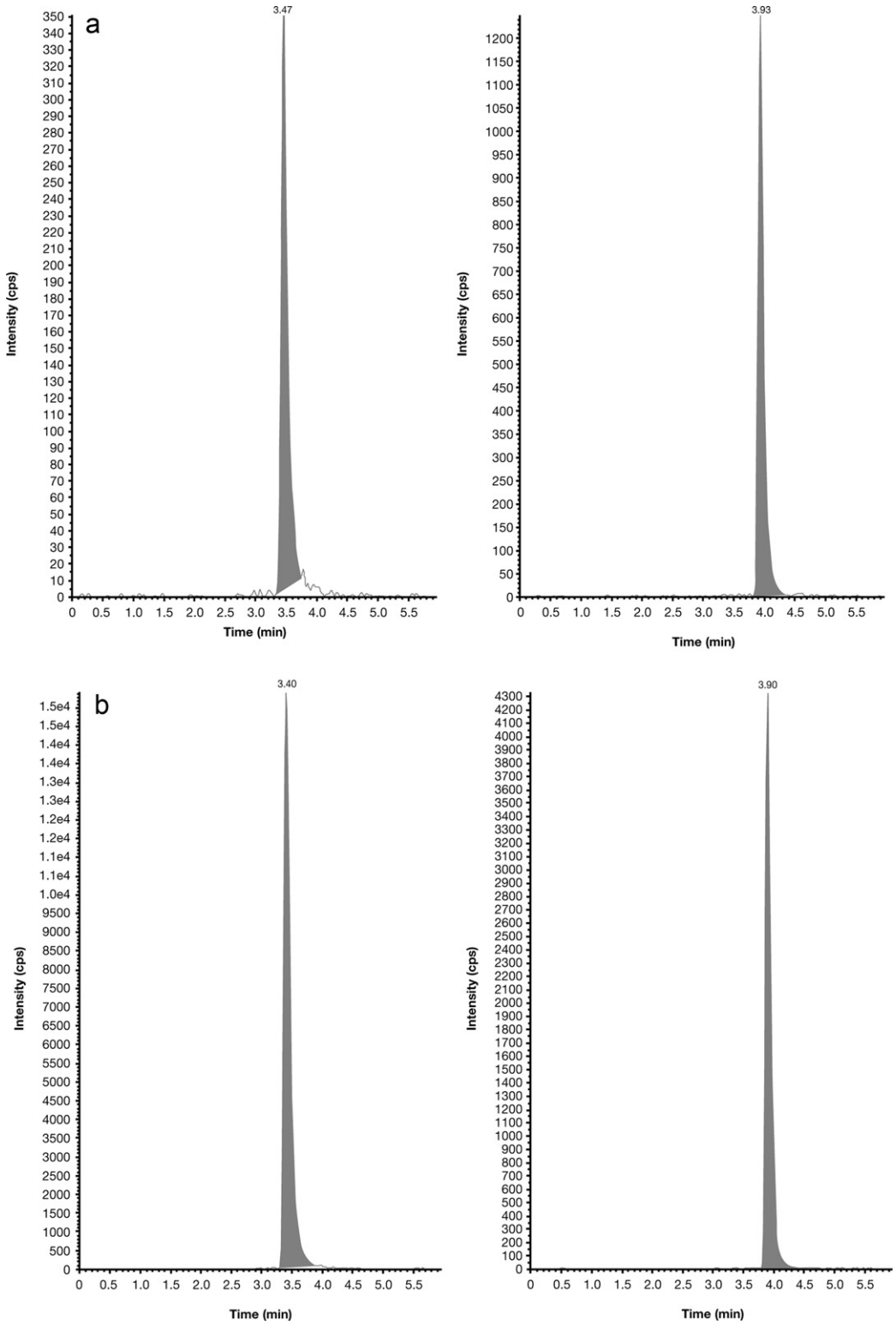

Fig. 4. Example chromatograms of sugammadex at (a) 5 μg/mL (LLOQ) and (b) 80 μg/mL, both with the internal standard Org 26265 20 μg/mL in human urine extract. In each case, the chromatograms on the left show sugammadex, while the chromatograms on the right show the internal standard.

For example, in a recent phase I study of the effects of sugammadex doses up to 32 mg/kg alone or in combination with rocuronium or vecuronium on QTc prolongation in healthy volunteers [9], more than 2600 plasma samples were analyzed in a total of 49 analytical runs, with only six runs failing to meet the acceptance criteria. For sugammadex, mean inter-run accuracy of the QC sample data was −5.0% (CV 10.9%, $n = 129$) at 0.300 μg/mL, −2.5% (CV 9.8%, $n = 129$) at 4.00 μg/mL, −0.6% (CV 10.3%, $n = 128$) at 32.0 μg/mL, 0% (CV 8.1%, $n = 128$) at 320 μg/mL and 1.3% (CV 7.9%, $n = 129$) at 1600 μg/mL, including outliers.

As quality control samples may not adequately mimic study samples from dosed subjects (incurred samples), reanalysis of incurred samples provides additional information to the evaluation of assay reproducibility. The method proved to be sufficiently

Table 4
Ion suppression from human urine.

	[Sugammadex] 15 μg/mL	[Internal standard] 20 μg/mL	Peak area ratio	Matrix factor (IS normalized)
	Peak area	Peak area		
Extracted samples				
	26,046	40,283	0.647	0.886
	27,211	43,282	0.629	0.861
	26,019	38,774	0.671	0.919
	27,433	41,385	0.663	0.908
	25,783	39,863	0.647	0.886
	25,605	38,304	0.668	0.916
Mean	26,350	40,315	0.654	0.896
%CV				2.5
Reference solution				
	37,989	52,114	0.729	
	39,186	52,170	0.751	
	38,538	52,602	0.733	
	36,939	51,679	0.715	
	38,297	52,299	0.732	
	38,046	52,848	0.720	
Mean	38,166	52,285	0.730	
Matrix effect (ion suppression)	31.0%	22.9%		

reproducible and incurred samples ($n = 180$) were found to generate consistent results for plasma samples from healthy volunteers. For plasma, 90% of the incurred samples had a relative bias of the initial value and the reanalysis value from the mean, calculated as $(2 \times (\text{initial value} - \text{reanalysis value})/(\text{initial value} + \text{reanalysis value})) \times 100\%$, of less than 30%, whereas the mean relative bias was −3.2% with a 90% confidence interval of ±2.27%.

Similarly, the assay to determine total sugammadex concentrations in urine has also been successfully applied in several clinical studies. For example, in a phase I study to assess and compare the safety, tolerability and pharmacokinetics of three ascending intravenous bolus doses with sugammadex in Japanese and Caucasian healthy male and female volunteers, 588 urine samples were analyzed over ten analytical runs, with none failing to meet acceptance criteria. One run was injected twice due to instrument failure. For sugammadex, mean inter-run accuracy of the QC sample data was −7.3% (CV 7.5%, $n = 30$) at 15.0 μg/mL, −6.9% (CV 7.7%, $n = 30$) at 80.0 μg/mL, −6.0% (CV 7.8%, $n = 30$) at 150 μg/mL, and −10.7% (CV 6.4%, $n = 9$) at 1500 μg/mL (Merck Sharp & Dohme Corp., data on file). Incurred sample reproducibility was not investigated for this assay in human urine.

The assay to determine total sugammadex concentrations in dialysate has also been successfully applied in one clinical study evaluating the dialysability of the sugammadex–rocuronium complex in subjects with severe renal impairment (NCT00656799). In total, 99 dialysate samples were analyzed over five analytical runs, with only one run failing to meet acceptance criteria. For sugammadex, the mean inter-run accuracy of the QC sample data was 9% (CV 7.3%, $n = 10$) at 0.300 μg/mL, 7.5% (CV 5.5%, $n = 10$) at 2.00 μg/mL, and −2.7% (CV 5.0%, $n = 10$) at 40.0 μg/mL. The method proved to be sufficiently reproducible and incurred samples ($n = 50$) of 6 subjects were found to generate consistent results for dialysate samples from renal impaired patients. For dialysate, 70% of the incurred samples had a relative bias from the mean, calculated as $(2 \times (\text{initial value} - \text{reanalysis value})/(\text{initial value} + \text{reanalysis value})) \times 100\%$, of <20% (Merck Sharp & Dohme Corp., data on file).

3.4. Discussion

The methods described here for the quantification of total sugammadex in human plasma, urine, and dialysate samples have been developed and validated according to FDA guidelines on bioanalytical method validation [10]. In clinical practice sugammadex is used to reverse the neuromuscular blockade induced by the NMBAs rocuronium and vecuronium, and as such clinical samples may contain both sugammadex and the NMBA. Due to the high binding affinities of rocuronium and vecuronium with sugammadex of $1.79 \times 10^7\ M^{-1}$ and $5.72 \times 10^6\ M^{-1}$, respectively [11], these NMBAs are encapsulated by sugammadex. As a consequence, sugammadex will be present both in unbound form and bound to NMBA. During sample processing and analysis, the sugammadex–NMBA complexes are disrupted [8], and as such the assays presented here will yield total sugammadex concentrations (i.e. both bound and unbound).

Prior to mass spectrometric detection, samples were subjected to sample pre-treatment and liquid chromatography. For human plasma and urine samples, pre-treatment was performed using solid-phase extraction, whereas dialysate samples were simply diluted with water. To stabilize sugammadex in urine after sample collection and during storage, heparinized plasma was added to urine samples within 1 h of collection. The addition of human plasma to urine in a 1:1 ratio also improved the ruggedness of solid-phase extraction, as without the addition, inconsistent results were obtained when applying solid-phase extraction to plain human urine samples.

Due to the metal binding affinity of sugammadex, it was essential to avoid contact with metal as much as possible during sample processing and analysis. This was achieved by using PEEK columns and tubing during liquid chromatography. Extensive rinsing of the injection syringe was also performed to counteract the absorption of trace amounts of sugammadex. Nevertheless, carry-over could not always be eliminated. To monitor for the occurrence of carry-over, blank samples were injected after the highest calibration sample and after the highest QC-sample. If carry-over was detected, the impact on sample analysis was evaluated and where necessary, re-analysis was performed.

Dilution integrity was demonstrated for each matrix. For heparinized human plasma, a 50-fold dilution was validated, whereas 10-fold and 100-fold dilutions met the acceptance criteria for accuracy and precision for human urine and dialysate, respectively. Together with the established lower limits of quantification, dilution enabled the analysis of clinical samples within the range relevant to clinical practice.

Although ion-suppression from plasma and urine to both sugammadex and internal standard was demonstrated, the variation of

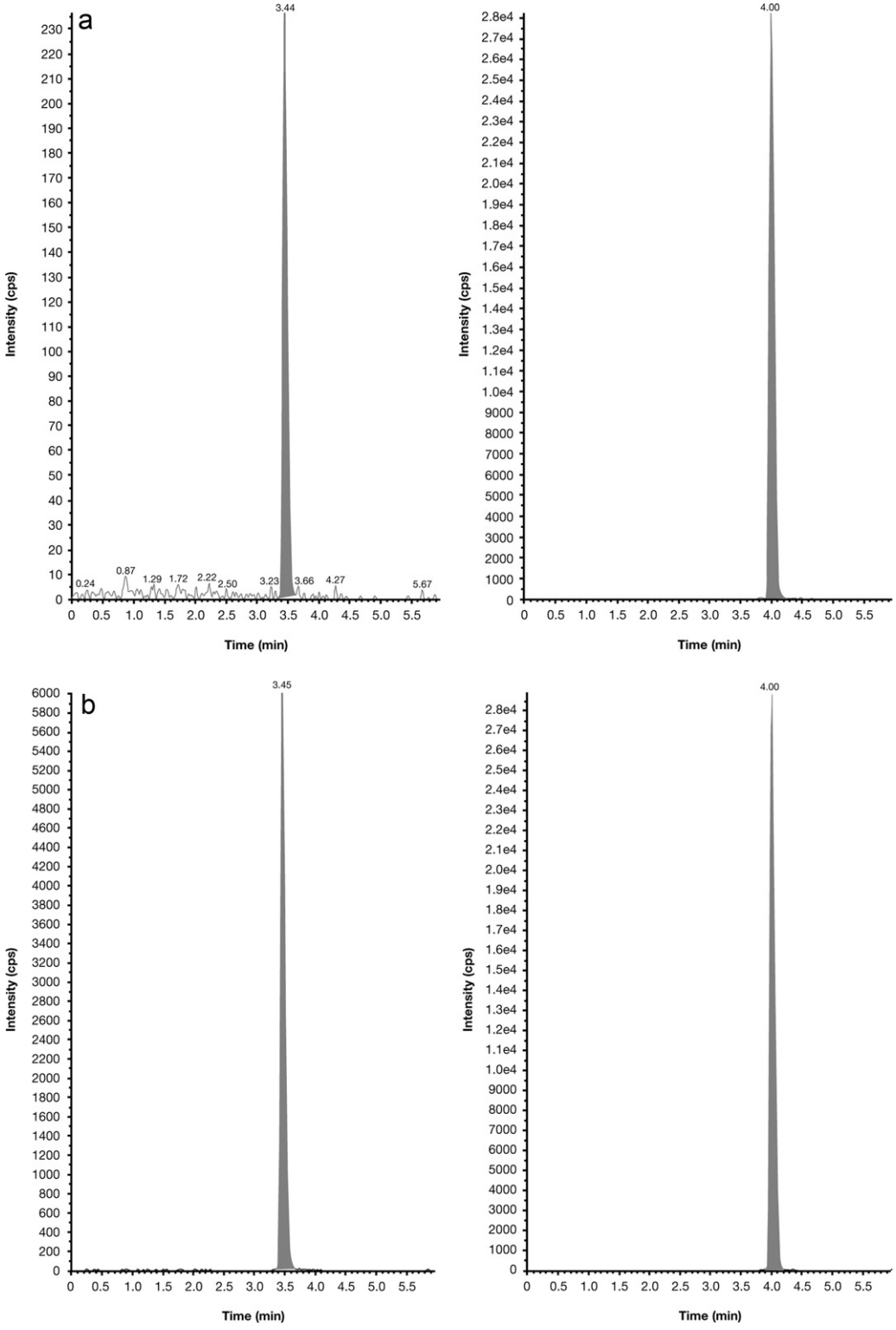

Fig. 5. Example chromatograms of (a) sugammadex 0.1 μg/mL (LLOQ) and (b) sugammadex 2 μg/mL, both with the internal standard Org 26265 15 μg/mL in human dialysate samples. In each case, the chromatograms on the left show sugammadex, while the chromatograms on the right show the internal standard.

the internal standard normalized matrix effect for different matrix lots was within acceptable limits.

Long and short-term sample stabilities were key considerations for the assay. Plasma, urine, and dialysate samples were all found to be stable in the short-term at room temperature (for at least 24 h for plasma and dialysate samples, and at least 72 h for urine samples) as well as after long-term storage at −20 °C and after several freeze–thaw cycles. Sugammadex was also found to be stable in human urine samples at 37 °C for at least 1 h, which is important due to the nature of urine sampling in clinical trials.

These assays, developed for the determination of total sugammadex in plasma, urine, and dialysate, showed no significant interference from exogenous and/or endogenous compounds during assay validation. As sugammadex is used clinically as an agent

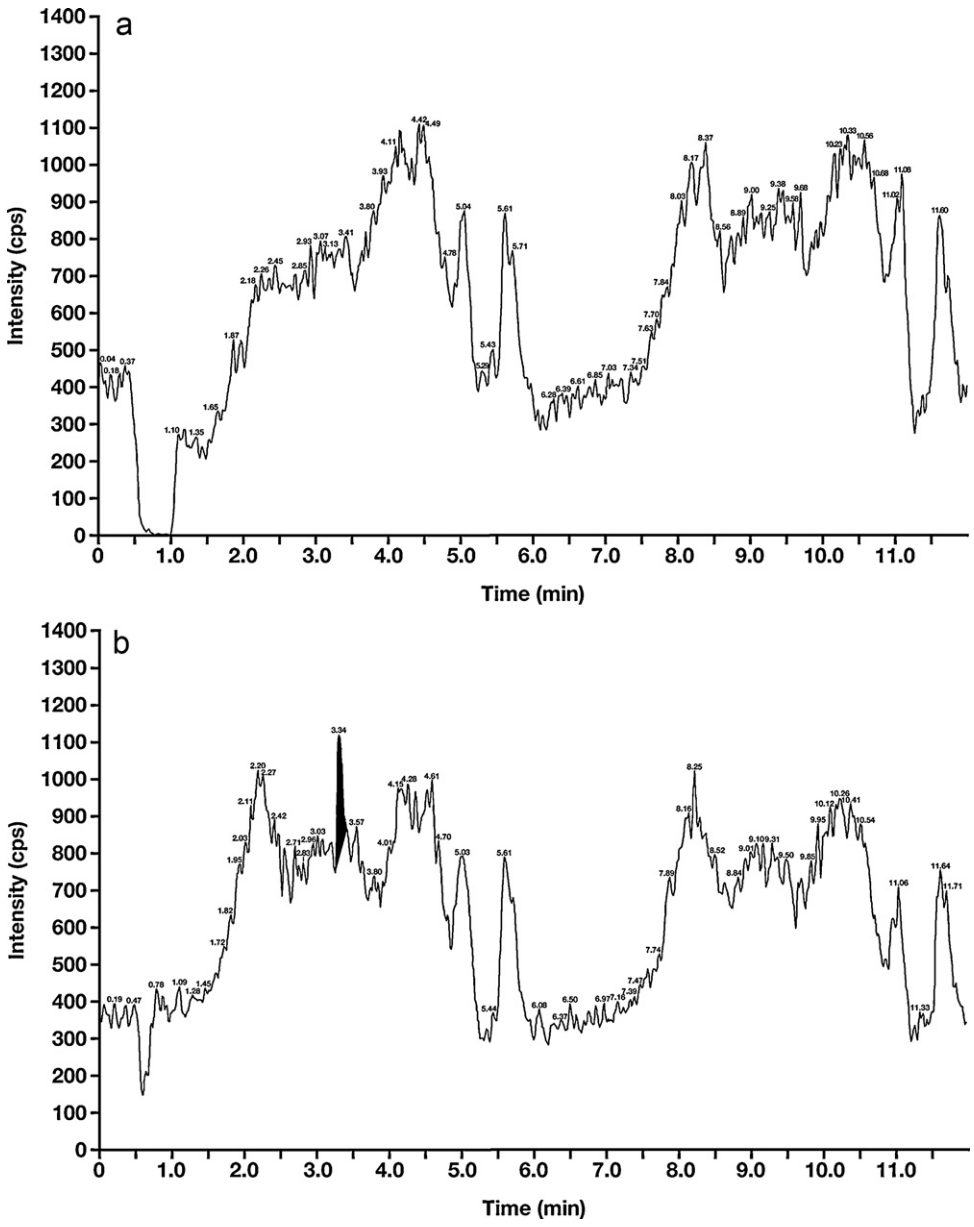

Fig. 6. Chromatograms of matrix effect investigations, with post column addition of (a) an aqueous solution of 1 ng/mL sugammadex when blank plasma was injected and (b) when an aqueous solution of 15 ng/mL sugammadex was injected.

to reverse neuromuscular blockade by encapsulating rocuronium or vecuronium, it was therefore important to elucidate whether the presence of either rocuronium or vecuronium had an impact on the quantification of sugammadex. QC samples spiked with rocuronium at the anticipated maximal plasma concentration of rocuronium were found to be within pre-defined accuracy criteria, indicating that the presence of rocuronium in the samples does not have an influence on the results for sugammadex. Similarly, QC samples spiked with vecuronium were also within pre-defined accuracy criteria. As the clinical trial program for sugammadex progressed, a limited number of additional compounds have been identified which based on their binding affinity for sugammadex might have the potential to interfere with the assay. For example, flucloxacillin has a moderate binding affinity (K_a) with sugammadex of $5.22 \times 10^4\,M^{-1}$ [11], and when given as concomitant administration at a high dose will give plasma concentrations of up to 1000 μg/mL. At this high concentration flucloxacillin was found to interfere with the assay in human plasma, thus preventing the determination of the total sugammadex concentration. Nevertheless, high-dose flucloxacillin in vivo does not cause reoccurrence of neuromuscular blockade after reversal with sugammadex [13].

The assay methods developed here have been successfully used for the determination of total sugammadex concentration in plasma, urine and dialysate in clinical studies, both when sugammadex was administered alone to volunteers [14,15] and when sugammadex was given to reverse rocuronium or vecuronium in volunteers and surgical patients [9,16–20]. This has enabled the pharmacokinetics of sugammadex and the sugammadex–NMBA complex to be further elucidated. Sugammadex has been found to be excreted almost exclusively via the renal route within 48 h with minimal to no metabolism, and typical plasma concentration and urinary excretion curves following administration of sugammadex 4 mg/kg are illustrated in Fig. 7. In this study, six healthy volunteers received ^{14}C-labelled sugammadex, allowing the geometric mean total concentration of radiolabelled sugammadex

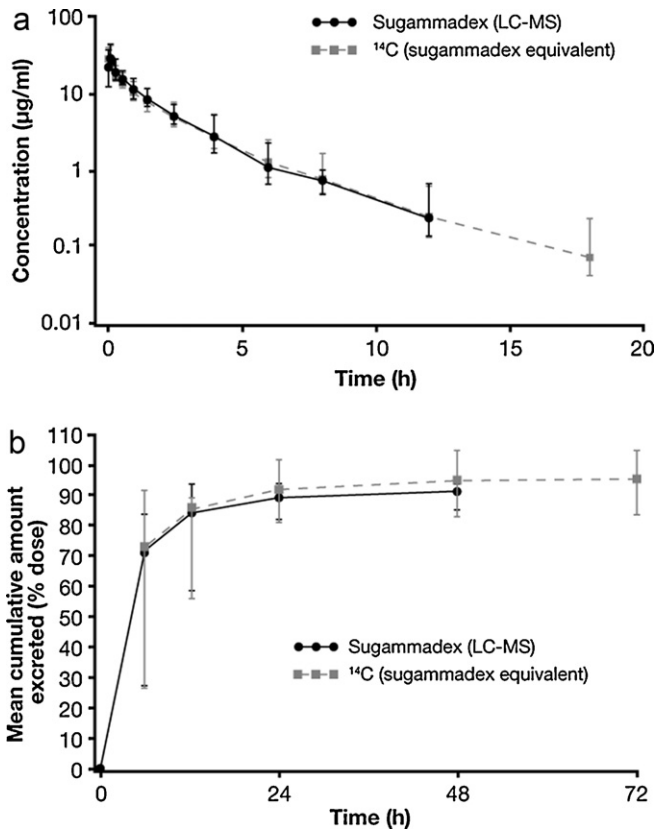

Fig. 7. (a) Geometric mean (range) sugammadex plasma concentration and (b) mean cumulative percentage urinary excretion, determined by LC–MS and ^{14}C-radioactivity following administration of a single intravenous 4 mg/kg dose of ^{14}C-labelled sugammadex to six healthy volunteers [21].

determined by LC–MS to be compared with total ^{14}C-radioactivity. The overlapping curves provided further validation of the LC–MS assay [21].

In summary, validated bioanalytical methods have been developed for the determination of the total concentration of sugammadex, comprising free sugammadex and the sugammadex–NMBA complex in human plasma, urine and dialysate. These assays will be especially important as clinical evaluation of sugammadex continues, allowing full characterization of the pharmacokinetics of sugammadex.

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