RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2002; **16**: 1946–1952
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.812

Liquid chromatography/mass spectrometric bioanalysis of a modified γ-cyclodextrin (Org 25969) and Rocuronium bromide (Org 9426) in guinea pig plasma and urine: its application to determine the plasma pharmacokinetics of Org 25969

Ola Epemolu[1]*, Iain Mayer[2], Frank Hope[1], Paul Scullion[2] and Paul Desmond[2]

[1]DMPK Section, Department of Pharmacology, Organon Research, Newhouse Industrial Estate, Lanarkshire ML1 5SH, Scotland, UK
[2]DMPK Section, Department of Analytical Chemistry, Organon Research, Newhouse Industrial Estate, Lanarkshire ML1 5SH, Scotland, UK

Received 31 May 2002; Revised 8 August 2002; Accepted 8 August 2002

A sensitive and specific liquid chromatography/mass spectrometry (LC/MS) method has been developed and validated for the quantification of the modified γ-cyclodextrin Org 25969 and Rocuronium bromide (Roc or Org 9426) in the plasma and urine of guinea pigs. The assay was linear and reproducible over the range 25–10000 ng/mL for both compounds. The lowest limit of quantification (LLOQ) for both compounds in urine was 25 ng/mL. In plasma, the LLOQ was 25 ng/mL for Org 9426 and 50 ng/mL for Org 25969. The inter- and intra-day variation was lower than 20%. The physicochemical properties of both compounds imposed different modes of extraction from plasma. The modified γ-cyclodextrin was extracted by trifluoroacetic acid (TFA) precipitation while Rocuronium was extracted by acetonitrile precipitation. Both compounds were quantified in urine by direct injection onto the column. The LC/MS analyses of Org 25969 and Org 9426 were performed using two different assay conditions. It was not possible to quantify the complex of cyclodextrin and Roc as it dissociated on the LC column. The use of LC/MS conferred great advantage to the quantification of both Org 25969 and Org 9426, as they were not chromogenic enough to afford the sensitivity and specificity required for the assay. Copyright © 2002 John Wiley & Sons, Ltd.

Org 25969 is a novel, modified γ-cyclodextrin derivative with high affinity for neuromuscular blocking agents (NMBAs) such as Roc (See Fig. 1). Org 25969 is currently in clinical phase I development as an NMBA reversal agent. Reversal agents are used to reverse non-depolarising neuromuscular block in order to speed up recovery of neuromuscular function. In addition, they can also be used when residual neuromuscular block occurs, which is often associated with post-operative pulmonary complications.[1]

Traditionally, the effect of non-depolarising neuromuscular blocking agents (NMBAs) is reversed by the administration of an acetylcholinesterase inhibitor. Inhibition of acetylcholinesterase in the neuromuscular junction increases the survival time of acetylcholine in the synaptic cleft. The increase in the level of acetylcholine enables it to outcompete the NMBA for binding to the nicotinic acetylcholine receptor.

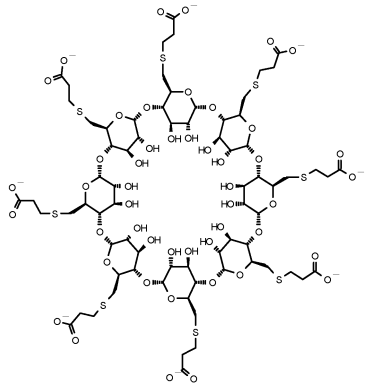
**Correspondence to*: O. Epemolu, DMPK Section, Department of Pharmacology, Organon Research, Newhouse Industrial Estate, Lanarkshire ML1 5SH, Scotland, UK.
E-mail: o.epemolu@organon.co.uk

Inhibition of acetylcholinesterase causes an increase in acetylcholine levels in all cholinergic synapses, resulting in undesired stimulation of muscarinic and nicotinic acetylcholine receptors in other tissues, e.g. the smooth muscles in the respiratory and gastro-intestinal tract. Pre-treatment with muscarinic receptor antagonists, like atropine or glycopyrrolate, can be used to reduce the unwanted stimulation of muscarinic acetylcholine receptors. However, administration of these drugs is contra-indicated in certain patients.

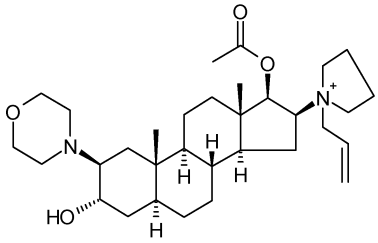
Acetylcholinesterase inhibitors also have other disadvantages as they produce a long-lasting inhibition of the enzyme and cannot be used to reverse profound neuromuscular block.[2]

In clinical practice, administration of acetylcholinesterase inhibitors after pre-treatment with muscarinic antagonists has been used successfully for many years for reversal of neuromuscular block, but this method of treatment is far from ideal.

Therefore, a clear need exists for a novel reversal agent that lacks the disadvantages of the acetylcholinesterase inhibitors. Ideally, this agent would lack any action on receptors or enzymes involved in neuromuscular trans-



Org 25969



Org 9426 (Rocuronium®)

Figure 1. Chemical structures of Org 25969 and Org 9426.

mission, would not require pre-treatment with muscarinic antagonists, and would be able to reverse profound block.

Org 25969 meets all these criteria and, as part of early pre-clinical development, it became apparent that a sensitive and specific method was required to quantify Org 9426 and Org 25969 in both plasma and urine of guinea pigs.

The immediate challenge was how to simultaneously or sequentially quantify Org 9426, Org 25969 and their 1:1 complex in plasma or urine. Org 25969 is a complex hydrophilic sugar while Org 9426 is a lipophilic organic moiety. The calculated Log P (Clog P) for Org 25969 and Org 9426 were −19.23 and 2.44, respectively.

A survey of the scientific literature on the quantification of cyclodextrins showed a paucity of methods with good sensitivity; typically LOQs were not less than 4μg/mL. There were no reported attempts to quantify cyclodextrins in the presence of another compound apart from the work of Grosse *et al.*[3] who resorted to using two HPLC methods with fluorimetric detection. As a consequence of the difficulty in quantifying cyclodextrins, some authors have attempted HPLC with refractive index,[4–7] pulsed amperometric,[8] indirect photometric,[9] evaporative light scattering[10] and radio-detection methods with limited success.[11] Others have investigated the ability of cyclodextrins to form inclusion complexes with limited success regarding sensitivity.[3,12] More recently, Hammes *et al.*[13] investigated the use of LC/MS in positive selected-ion mode monitoring (SIM) with improved sensitivity.

This paper describes the development and validation of a sensitive and specific LC/MS method for the determination of Org 25969 (a modified γ-cyclodextrin) and Org 9426 (Rocuronium) in the plasma and urine of guinea pigs. The assay was performed in the SIM mode for Org 25969 and in the multiple reaction monitoring (MRM) mode for Org 9426. The assay has been applied to establish the pharmacokinetics of Org 25969 in guinea pigs that were undergoing neuromuscular block following intravenous infusion of Org 9426.

EXPERIMENTAL

Materials

Org 25969 was synthesised as described by Bom *et al.*[14] and Org 9426 was synthesised in The Department of Medicinal Chemistry, Organon Research, Scotland. The purity of both compounds was ca. 99%. All other chemicals and reagents were analytical or HPLC grade. Acetonitrile was obtained from BDH (Poole, UK); ammonium acetate, formic acid and trifluoroacetic acid were purchased from Fisher Scientific (Loughborough, UK).

Instrumentation

Org 25969

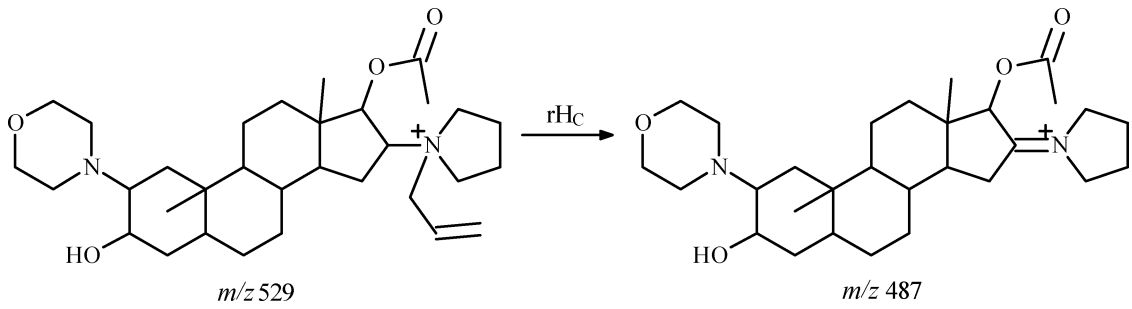
The LC/MS method used a Sciex 3000 mass spectrometer equipped with turbo-ion spray (T-ISP) at 400 °C in the negative ion mode with Perkin Elmer series 200 micro-LC pumps, Perkin Elmer series 200 autosampler, column oven and 6-port switching valve. The chromatographic conditions were as follows: the column employed was a Jupiter C18 (150 × 4.6 mm, 5 μ) from Phenomenex (Cheshire, UK); the flow rate was 2.0 mL/min with a split ratio of 10:1 waste: MS; the solvents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); the gradient was 100% (A) for the first 2.0 min dropping to 50% by 12 min. This was then dropped to 0% (A) for the next 2.0 min before equilibration for a further 2.0 min at its initial composition. The run time was 16 min and the injection volume was 10 μL.

The Turbo gas flow was set to 6 L/min. The ions monitored in the SIM experiment for Org 25969 and Org 25289, γ-cyclodextrin (internal standard) were *m/z* 1000 (doubly charged ion) and *m/z* 1342 (due to the presence of the formate adduct), respectively. The dwell time was 200 ms and the tuning conditions were optimised for each compound separately by infusion of a 1-μg/mL solution.

Org 9426

The column employed for Org 9426 was a Jupiter C4 (50 × 4.6 mm, 5 μ) from Phenomenex (Cheshire, UK); the flow rate was 1.3 mL/min with a split ratio of 10:1 waste: MS; the solvents were 20 mM ammonium acetate (A) and acetonitrile (B); the gradient was 90% (A) for the first 1.0 min dropping to 10% by 2.5 min. This was maintained for the next 1.0 min before being allowed to equilibrate for a further 0.5 min at its initial composition. The run time was 4 min and the injection volume was 10 μL.

The interface temperature was 300 °C and the ionisation source was the turbo-ion spray (T-ISP) operated in positive ion mode. The Turbo gas flow was set to 6 L/min. The multiple reaction monitoring (MRM) transitions used for Org 9426 and Org 24748 (the internal standard) were *m/z* 529 to 487 (see proposed transition below) and *m/z* 310 to 91, respectively. The dwell time was 200 ms and the tuning



conditions were optimised for each compound by infusion of a 1 μg/mL solution.

Animal dosing

Male Dunkin-Hartley guinea pigs (Harlan, UK) weighing approximately 600–800 g were anaesthetised using urethane (900 mg/kg) and pentobarbitone sodium (30 mg/kg). The guinea pigs were ventilated with ambient air (0.75 mL/100g and 60 strokes/min).

Blood pressure/heart rates were monitored via cannulae placed in the left carotid artery. The gastrocnemius muscle contractions were elicited by stimulation via the sciatic nerve at 2× maximal voltage, at a frequency of 0.1 Hz; 0.25 ms pulse width. This was recorded to give an indication of the degree of muscle relaxation.

The vehicle used was phosphate buffer for injection (Department of Pharmacy, Organon NV, Oss, Netherlands). Org 9426 was administered as a bolus (0.09 mg/kg) via a cannula placed in the right jugular vein and flushed in with 0.2 mL saline. This was followed immediately by the infusion of 100 μg/mL Org 9426 at a rate titrated to obtain and maintain stable neuromuscular block of approximately 90% for 5 min. The mean infusion rate giving a stable 90% block of muscle twitch was 8.5 μg/kg/min.

Upon the achievement of stable 90% neuromuscular block, the Rocuronium infusion was stopped and a bolus dose of Org 25969 at 0.15, 0.5 or 1.0 mg/kg was then injected via the left jugular vein to commence the reversal of the block.

Arterial blood samples were collected at 0, 10, 20, 30, 40, 50 and 60 min and subsequently processed for plasma samples, which were stored at −20 °C until analysed. Corresponding urine samples were collected with the aid of an indwelling bladder catheter, which afforded a bladder rinse of 1 mL saline per collection time. The mean concentrations (n = 4) were then plotted against time for each dose of Org 25969.

Assay procedure

Plasma

Stock solutions of either Org 9426 or Org 25969 were prepared in 50:50 (v/v) acetonitrile/water and Milli-Q water, respectively, at concentrations of 0.1, 1.0, 10.0 and 100 μg/mL. From these solutions, 100 μL of blank plasma were spiked to achieve final concentrations in the range 2.5–10000 ng/mL.

Plasma containing Org 9426 was extracted by adding 300 μL of acetonitrile containing 40 ng/mL of the internal standard (Org 24748) to 100 μL of each plasma sample. The samples were vortexed and centrifuged at 2700 *g* to give clear supernatants that were aspirated using a disposable Pasteur pipette. The supernatants were concentrated down using a Savant Speedvac® sample concentrator (Thermo Life Sciences, Hants. UK).

To each 100-μL plasma sample were added 5 μL of a 10-μg/mL stock solution of the internal standard Org 25289. The precipitation was performed using 10 μL of neat TFA.

The TFA-extracted plasmas were then centrifuged as for the Org 9426 plasma. The resultant supernatants were injected onto the LC/MS column as described under Instrumentation.

Urine

The stock solutions were the same as described for the plasma. The urine calibration samples were prepared in similar fashion to plasma with the exception that for both Org 9426 and Org 25969, 5 μL of the appropriate internal standards (10 μg/mL) were spiked into the corresponding urine samples.

Following vortex mixing the samples were then injected directly into the LC/MS system.

Acceptance criteria for calibrations

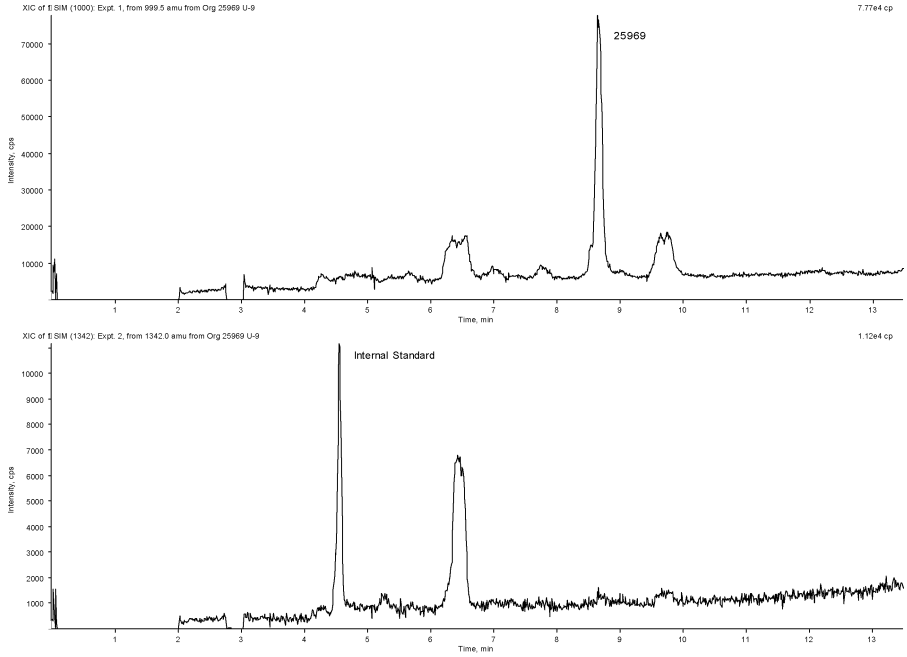
The calibrations were accepted if two set of calibrations were within ±20% of their expected values. Quality control samples (QCs) were prepared by another scientist and the same acceptance criteria were applied to them. The lowest acceptable QC level was used to set the lowest limit of quantification (LLOQ). The LLOQ for the determination of Org 9426 in plasma and urine was 25 ng/mL. The corresponding LLOQs for Org 25969 in plasma and urine were 50 and 25 ng/mL, respectively. The extraction efficiency of the plasma assays was not determined.

Precision and accuracy

Intra- and inter-day precision and accuracy were assessed by quadruplicate determination of Org 9426 and Org 25969 in both plasma and urine. The QC levels that were used were 25 or 50, 250 and 2500 ng/mL. The accuracy was defined as (observed concentration/expected concentration) ×100, while the precision was expressed as (SD/mean of observed concentration) ×100.

RESULTS

Figure 2 show typical LC/MS/(MS) chromatograms for Org 25969 and γ-cyclodextrin (Org 25289) from plasma. For all calibration curves, a weighting of 1/x was used. The LC/MS/(MS) chromatograms for the urine extract were similar.

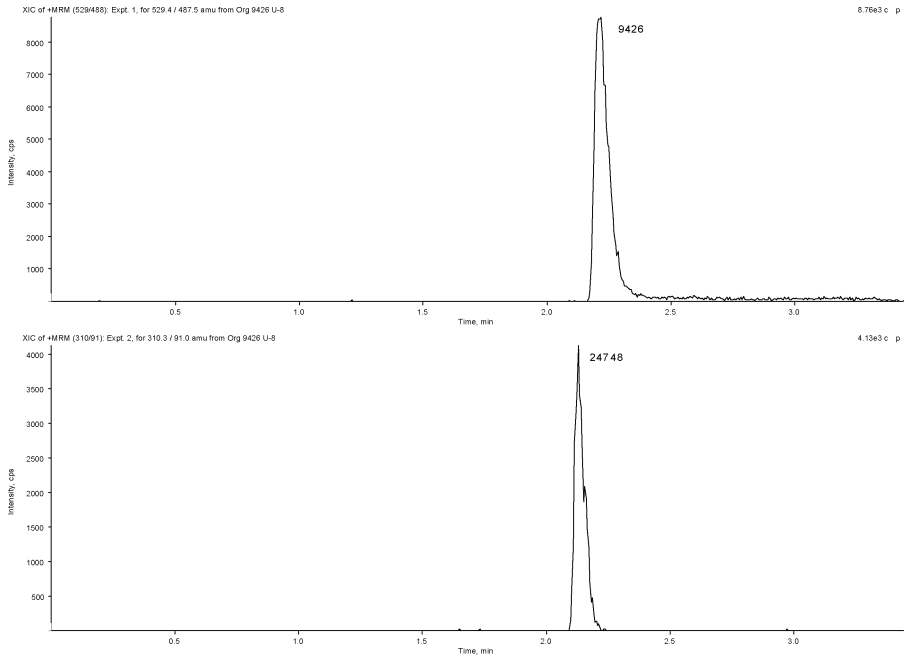


Upper panel : Extracted ion chromatogram (XIC) of 25969 at *m/z* 1000

Lower panel: Extracted ion chromatogram (XIC) of γ-cyclodextrin at *m/z* 1342

Figure 2. Extracted ion chromatograms (XIC) of Org 25969 (500 ng/mL) and γ-cyclodextrin in plasma extract.

There were no interfering peaks in either the plasma extract or blank urine for both analytes in both matrices. The calibration curves for Org 25969 in plasma and urine were satisfactory within the range 25–10000 ng/mL for urine and 50–10000 ng/mL for plasma (correlation coefficient of >0.99). Similar results were obtained for Org 9426 in both matrices. A typical chromatogram for Org 9426 in plasma is shown in Fig. 3. The urine chromatogram was similar. The



Upper panel : Selected reaction monitoring (SRM) chromatogram of Org 9426 *m/z* 529–487

Lower panel: Selected reaction monitoring (SRM) chromatogram of Org 24748 *m/z* 310–91

Figure 3. Selected reaction monitoring chromatograms (SRM) of Org 9426 (500 ng/mL) and Org 24748 in plasma extract.

Table 1. Inter-day precision and accuracy for Org 25969 in plasma and urine of guinea pigs

	Plasma		Urine	
Concentration (ng/mL)	Accuracy*	Precision**	Accuracy	Precision
25	ND	ND	95	9.2
50	103	6.1	88	7.5
250	106	15.8	106	6.2
2500	93	14.1	101	13.6

Key: Accuracy * is defined as observed mean concentration/nominal or expected concentration ×100, while the precision ** is expressed as SD/mean of observed concentration ×100. ND equates not determined.

Table 2. Inter-day precision and accuracy for Org 9426 in plasma and urine of guinea pigs

	Plasma		Urine	
Concentration (ng/ml)	Accuracy*	Precision**	Accuracy	Precision
25	102	9.4	88	6.7
250	107	6.4	93	2.8
2500	92	2.7	113	8.0

Key Accuracy * is defined as observed mean concentration/nominal or expected concentration ×100, while the precision ** is expressed as SD/mean of observed concentration ×100. ND equates not determined.

calibration curves for Org 9426 in plasma and urine were satisfactory within the range 25–10000 ng/mL for urine and 50–10000 ng/mL for plasma (correlation coefficient of >0.99).

The inter-day precisions for the assay are shown in Tables 1 and 2 and they were <20% of their expected or nominal values.

The stability of Org 25969 (data not shown) was investigated after three freeze-thaw cycles over 1 month. It was found to be stable in both matrices. The extraction efficiency of Org 25959 from plasma was not determined because of the matrix effect, which can preclude accurate determination.[13]

Figure 4 shows the plasma concentration against time profile for Org 25969 following the administration of 0.15, 0.5 and 1.0 mg/kg intravenous administration. The plasma levels declined in a bi-phasic fashion over the 1-h period. The corresponding pharmacokinetics (PK) parameters were calculated using WinNonlin® (Pharsight Inc, USA). The PK parameters are shown in Table 3. The effect of Org 9426 on the PK of Org 25969 was also investigated. The urinary profile of Org 25969 is shown in Fig. 5 and demonstrates dose-related increase in urinary excretion. The effect of Org 25959 on the urinary excretion of Org 9426 is shown in

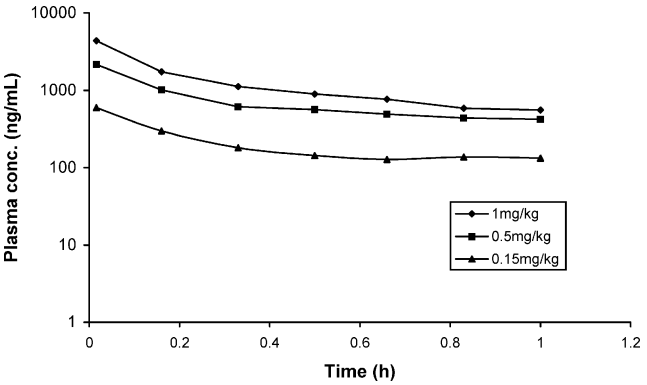


Figure 4. Plasma concentration against time profile for Org 25969 following intravenous bolus administration to anaesthetised and neuromuscularly blocked guinea pigs at various doses.

Table 3. Comparison of pharmacokinetic parameters of Org 25969 in the presence and absence of Rocuronium (Org 9426) after three different administrations of Org 25969 to anaesthetised guinea pigs

	Dose level (mg/kg)		
	0.15	0.5	1.0
$C_{initial}$ (ng/mL)	644 (1014)	2443 (4043)	4834 (8844)
AUC (ng/mL.h)	539 (492)	1450 (1703)	1755 (3090)
Cl (mL/h/kg)	278 (305)	345 (293)	570 (323)
$T_{1/2}$ (min)	104 (39)	71 (42)	38 (33)
Volume of distribution (Vz) (mL/kg)	698 (288)	586 (299)	522 (255)

Key: values in parentheses are in the presence of Org 9426 (Rocuronium).

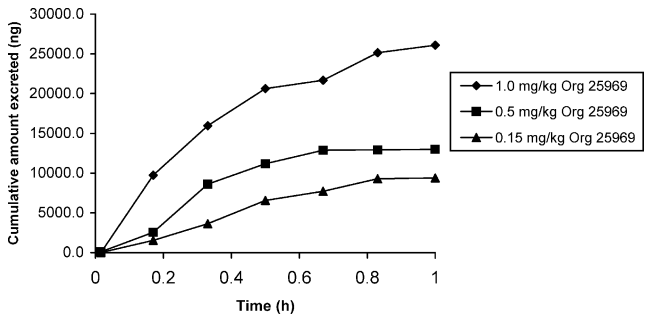


Figure 5. Mean cumulative amount excreted in urine for Org 25969 following intravenous bolus administration to anaesthetised guinea pigs at various doses.

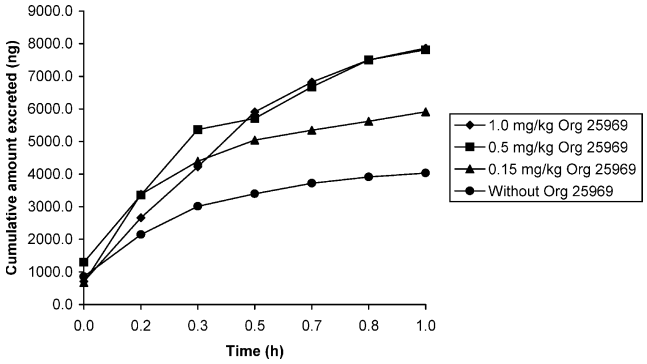


Figure 6. Mean cumulative amount excreted in urine for Org 9426 following intravenous bolus administration of Org 25969 to anaesthetised and neuro-muscularly blocked guinea pigs at various doses.

Fig. 6. It shows that Org 25969 does enhance the urinary excretion of Roc.

To determine the effect of one analyte on the other and to mimic the experimental situation, the plasma and urine calibration curves as well as the QC's were co-spiked with both Org 9426 and Org 25969 on the day of analysis and then subjected to the different extraction procedures as detailed under "Assay procedure". This investigation provided satisfactory results, as the determined LLOQ's remained the same for both analytes.

DISCUSSION

The SIM mode was employed for the quantification of Org 25959 and its corresponding internal standard, Org 25289. This mode was selected as it gave the highest signal intensity and therefore the best possible chance of achieving the sensitivity required for detecting both compounds. The MRM mode was investigated but Org 25969 showed minimal fragmentation in the API 3000 and was therefore not suitable. γ-Cyclodextrin was, in terms of chemical structure, the closest compound to Org 25969 that was readily available for use as an internal standard. For Org 9426 and its corresponding internal standard, Org 24748, the MRM mode was employed as it afforded the specificity needed to detect and quantify both compounds. Org 24748 has been used as a 'generic' internal standard for neuromuscular blockers.

The applicability of two specific and sensitive LC/MS/(MS) methods has been studied for the determination of the PK of Org 25969 and its ability to increase the urinary excretion of Org 9426 in anaesthetised and neuromuscularly blocked guinea pigs. The method was applied initially to determine the plasma PK of Org 25969 in anaesthetised guinea pigs, and in those that were anaesthetised and neuromuscularly blocked. The urinary excretion of both Org 25969 and Org 9426 has been studied using the reported assay.

The initial challenge of quantifying two compounds with dissimilar physicochemical characters has been circumvented by the use of two extraction procedures as well as two chromatographic conditions. The magnitude of this problem can be visualised by considering their physicochemical properties. On one side, there was Org 25969 with ClogP of −19.23 and molecular weight of 2002, compared with the corresponding values for Org 9426, which were 2.44 and 503, respectively. These problems collectively mitigated against the development of the assay and consequently imposed the need for two assay methods.

The problem of lack of chromophore was overcome by the sensitivity and specificity afforded by the use of the mass spectrometer. Attempts to use the same chromatographic conditions for both compounds failed because of their different physicochemical properties. The possibility of quantifying the complex of Org 25969 and Org 9426 was investigated; however, the complex dissociated under all liquid chromatographic conditions investigated.

The pharmacokinetics of Org 25959 are depicted in Fig. 4 and the corresponding parameters are given in Table 3.

The distribution of Org 25969 appears to have been reduced in the presence of Org 9426, as reflected by the values of Vz at the three dose levels. The initial concentration (C initial) of Org 25969 in plasma was almost doubled in the presence of Rocuronium indicating that Org 25969 was not being as rapidly distributed as it was when dosed alone. This was possibly due to protein binding or changes in the physicochemical characteristics of the complex, such that the reduced distribution resulted in an increase in C initial. Org 25959 showed dose-related urinary excretion. Subsequent toxicokinetic studies revealed that the renal elimination of Org 25959 was similar to that of creatinine clearance indicating that its clearance was mainly due to glomerular filtration (Diels van den Dobbelsteen, personal communication).[15]

In summary, it appears that the intravenous PK of Org 25969 was characterised by low distribution and short elimination half-life as observed for sulpho-butyl ether β-cyclodextrin.[16] However, in the presence of Rocuronium, the distribution appears to be decreased, suggesting that Rocuronium has the ability to influence the distribution of Org 25969.

CONCLUSIONS

The methods presented in this paper have been successfully applied to determine the plasma PK and urinary excretion of both Org 25969 and Rocuronium. This would not have been possible but for the use of LC/OMS/(MS) and a judicious use of extraction techniques. This method is now being applied to elucidate the toxicokinetics of Org 25969 in other species.

Acknowledgements

The contribution of Claire Mackie and Robin Parkinson to the PK analysis is appreciated.

REFERENCES

1. Berg H, Roed J, Viby-Morgensen J, Morgensen GR, Engbaek J, Skovgaard LT, Krintel JJ. *Acta Anaethesiol. Scan.* 1997; **41**: 1095.
2. Caldwell JE, Robertson EN, Baird WLM. *Br. J. Anaesth.* 1986; **58**: 1285.

3. Grosse PY, Bressolle F, Rouanet P, Joulia JM, Pinguet F. *Int. J. Pharmaceutics* 1999; **180**: 215.
4. Zsadon B, Otta KH, Tudos F, Szejtli J. *J. Chromatogr.* 1979; **172**: 20.
5. Schomburg G, Deege A, Hinrichs H, Hubinger E, Husmann H. *J. High Resolut. Chromatogr.* 1992; **15**: 579.
6. Koizumi K. *J. Chromatogr. A* 1996; **720**: 119.
7. White G, Katona T, Zodda JP, Eakins MN. *J. Chromatogr.* 1992; **625**: 157.
8. Koizumi K, Kubota Y, Tanimoto T, Okada Y. *J. Chromatogr.* 1988; **454**: 303.
9. Takeuchi T, Murayama M, Ishii D. *J. High Resolut. Chromatogr.* 1990; **13**: 69.
10. Caron I, Elfakir C, Dreux M. *J. Liq. Chromatogr. Relat. Technol.* 1997; **20**: 1015.
11. De Bie ATHJ, Van Ommen B, Bar A. *Reg. Toxicol. Pharmacol.* 1998; **27**: 150.
12. Gage R, Venn RF, Bayliss AJ, Edgington AM, Roffey SJ, Sorrel B. *J. Pharm. Biomed. Anal.* 2000; **22**: 773.
13. Hammes W, Bourscheidt C, Buscher U, Stodt G, Bokens H. *J. Mass Spectrom.* 2000; **35**: 378.
14. Bom A, Bradley M, Cameron K, Clark JK, van Egmond J, Feilden H, MacLean EJ, Muir A, Palin R, Rees DC, Zhang M-Q. *Angew. Chem.* 2002; **114**: 275.
15. Dobblesteen D. *NV. Organon Internal Report*, 2002.
16. Roffey SJ, Gibson D. *Drug Metab. Rev.* 2000; **32**: 96.