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ORIGINAL RESEARCH

The effect of cold-chain re-introduction on the molecular integrity of rocuronium bromide

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Introduction: This study aimed to examine the effect of breakages and re-introduction into cold chain on the rocuronium bromide compound. Rocuronium bromide is frequently used in routine theatre lists and plays a vital role in modified rapid sequence induction and intubation for emergency patients who have contraindications to the primarily used muscle relaxant, succinylcholine. With the current practice of removing the drug from, and then reintroducing it into the cold chain, unpredictable clinical effects, including delayed onset of action and shortened duration of action have been observed. This may pose significant risks to the patient.
Methods: Rocuronium bromide was subjected to different clinically applicable storage and temperature scenarios, after which the compound was analysed for integrity and quantities of the active compound, including detection of possible degradation products, by mass spectrometry, and compared to cold chain control samples.
Results: There were no significant differences between any of the temperature exposure groups (18 °C or 24 °C) or between single or double exposures at these temperatures. No statistically significant difference could be demonstrated between the two control groups (cold chain preserved and room temperature controlled) with testing done at weeks one and six. However, week twelve analysis revealed a statistically significant result which translates to a 26 µg/ml difference, which clinically would have no effect. Substantial results were obtained with a secondary exposure to air; which lead to a 20% decrease in rocuronium concentration ($p = 0.02$).
Conclusion: Practice should be adapted by keeping careful documentation as to when cold-chain was broken, and when the recommended 12 week period will lapse. Vial sharing as a standard is not recommended. If small quantities are repeatedly withdrawn from the vial during a prolonged case, the unused contents should be discarded after eight hours.

Keywords: rocuronium bromide, cold chain, liquid chromatography, mass spectrometry

Introduction

Rocuronium, an aminosteroid non-depolarising muscle relaxant, forms an important part of a balanced anaesthetic plan, and is often utilised for rapid sequence induction and intubation.[1-3] Rocuronium should be kept in a maintained cold-chain of 2-8 °C, and once removed may be kept unopened at sub-room temperatures for up to 60 days.[4,5] In clinical practice, sealed unused rocuronium vials are often returned to the refrigerator after a theatre list, on the assumption that the cold-chain was maintained in theatre.

However, pharmaceutical literature recommends that once rocuronium has been removed from the refrigerator, *it may not be returned*. Also, after breaking the vial's seal, the solution is reported to be stable for a period of 24 hours. However, lacking a preservative, any unused portions remaining in the vial should be discarded.[5-7]

In routine clinical practice, it is common that at the start of the day, a quantity of various muscle relaxants, including rocuronium and succinylcholine, are taken from the refrigerators and placed into small cooler boxes, which are then allocated to each operating theatre. However, with the frequent opening of the container and exposure to ambient theatre temperatures, the ice packs rarely stay frozen until the end of lists. Emergency theatres running 24h shifts have an even greater risk of temperature fluctuation in the cooler boxes.

Great variation in the pharmacological properties of muscle relaxants – specifically significantly delayed onset of action and shortened duration of action of rocuronium is commonly observed. These variations are random, resulting in unpredictable clinical manifestations during the conduct of anaesthesia. As rocuronium is a popular choice for elective sequence inductions as well as a commonly used alternative muscle relaxant for modified rapid sequence inductions, the effect of non-adherence to cold chain prescription is of clear relevance.[8]

The purpose of this study was to investigate the molecular effects – if any – of reintroducing rocuronium into the cold-chain.

Methods

Study design and setting

This comparative chemical analytic study of four experimental and two control groups was conducted in the Department of Chemistry at the University of Pretoria. The analysis utilised ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). This method provided the accurate mass of all compounds in the various rocuronium samples, including the mass values (Da) and

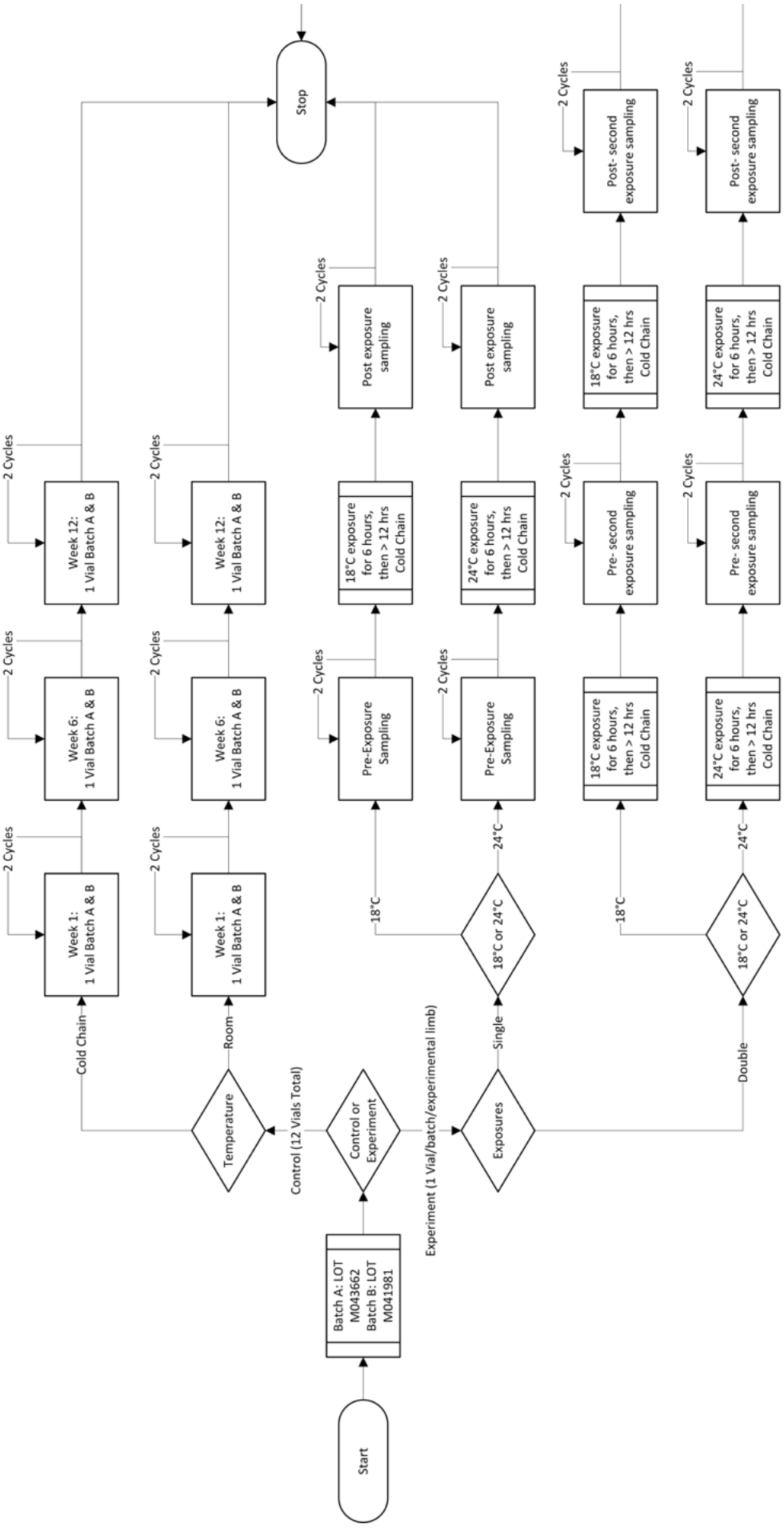


Figure 1: Flow chart of testing strategy

quantities of any degradation products. The accurate masses were then used to separate and identify these compounds.

Sampling preparation

Prior to commencing the research, two UTrix16 Multi-Use Universal Serial Bus (USB) Portable Document File (PDF) Temperature LogTag® Recorders (LogTag Recorders SA, Somerset West, South Africa) were issued to the pharmacy to record the cold-chain integrity of the shipment from the depot throughout the transport process. The LogTag units were configured to record temperatures every 10 minutes from start-up. Upon receipt of experimental stock, cold-chain integrity was verified and confirmed to be complete prior to the collection of specimens.

Two cartons of Esmeron® (Rocuronium Bromide, MSD South Africa, Halfway House) with LOT numbers M043662 and M041981, and expiry dates of June 2019 and March 2019, respectively were collected. These two batches were labelled Batch A (LOT M043662) and Batch B (LOT M041981).

Six vials of rocuronium bromide were selected at random, from nonspecific positions within the cartons from each batch.

Three vials from each batch were removed from the cold box and transferred directly into the laboratory cold chain refrigeration with their respective monitor for the duration of the 12-week study period in order to maintain cold chain conditions. This group was designated Control Group 1.

Three vials from each batch were removed from the cold box, packed together in an open container, and placed on a sun-protected shelf in the laboratory along with their respective monitor for the duration of the 12-week study period, in order to reflect the manufacturer's instructions of storing rocuronium unrefrigerated, below 30 °C, and discarding within 12 weeks once removed from the cold-chain. This group of vials was designated Control Group 2.

In order to test the effects of re-introducing rocuronium into the cold chain after exposure to various clinical temperatures, a randomly selected vial from each batch was submerged in temperature-controlled water baths for six hours in the laboratory. Bath temperatures of either 18 °C or 24 °C were maintained and recorded using a thermo-couple temperature probe or a mercury thermometer, which demonstrated a measurement accuracy of less than 0.5 °C variance. After the allotted submersion time in the respective temperature baths were completed, the single exposure group specimens were returned to the cold-chain refrigerator for a period exceeding 12 hours prior to final testing and analysis. These results were compared to a preliminary analysis which was conducted prior to the water bath exposure, using a standardised specimen preparation technique.

In order to assess if repeated exposures to environmental temperatures for 24 hours limits rocuronium's integrity, a double exposure group, was randomly selected from batches A and B. These specimens were exposed to the temperature variables by immersing them in water baths of 18 °C or 24 °C for six hours and then returning them to the cold-chain for 24 hours. This was followed by a pre-secondary exposure analysis, a second period of required temperature exposure, and another 12-hour post exposure return to the cold-chain prior to their final analysis. (Figure 1)

After sample preparation, each specimen was tested twice to yield two data points per sample per batch.

Sample preparation

A total volume of 100 µl rocuronium bromide was aspirated from the sealed vial using a BD (Becton Dickinson South Africa, Gauteng) Micro-Fine Plus Insulin Syringe® with a 29 Gauge (G) needle placed at an acute angle through the rubber stopper to prevent entrainment of air and was transferred into a clean MN Mass Spec® Glass Testing Vial. The sample was diluted by adding 900 microliter (µl) 50% solution of acetonitrile and water yielding a final concentration 1 000 microgram/millilitre (µg/ml) rocuronium bromide followed by five seconds of mixing using a vortex agitator. This solution was further diluted, using a BD Blunt Fill Needle® with 5-micron filter, and a SurgiPlus® (Surgi Plus, Westville) 1 ml luer slip syringe to prevent any debris contamination. The total aspirated volume of 100 µl was diluted with 900 µl acetonitrile and water solution to 100 µg/ml.

Lastly, from this 100 µg/ml solution, 100 µl was pipetted out, and diluted with 900 µl of acetonitrile and water solution, yielding a final 10 µg/ml concentration of rocuronium, or 10 parts per million. This final concentration was used for the analysis.

UHPLC-QTOF-MS method

Reagents

Ultra-purity liquid chromatography mass spectrometry (LCMS) grade water and acetonitrile (Romil-UpS™, Microsep, South Africa) were used for UHPLC-QTOF-MS analysis. Formic acid (99+% purity) (Thermo Scientific, South Africa) and ammonium hydroxide ≥ 25% in H_2O, eluent additive for LC (Fluka® Analytical, Sigma-Aldrich, South Africa) were used as buffers.

Instrument description

Compound separation and detection were performed using a Waters® Synapt G2 high definition mass spectrometry (HDMS) system (Waters Inc., Milford, Massachusetts, USA). The system comprises a Waters Acquity ultra performance liquid chromatography (UPLC®) system hyphenated to a quadrupole time-of-flight (QTOF) instrument. The system was operated with MassLynx™ (version 4.1) software (Waters Inc., Milford, Massachusetts, USA) for data acquisition and processing. An internal lock mass control standard, 2 picogram/microlitre (pg/µL) solution leucine enkephalin (*m/z* 555.2693), was infused directly into the source through a secondary orthogonal electrospray ionisation (ESI) probe allowing intermittent sampling. The internal control was used to compensate for instrumental drift, ensuring good mass accuracy, throughout the duration of the runs. The instrument was calibrated using sodium formate

clusters and Intellistart functionality (mass range 112.936–1 132.688 Dalton [Da]). Resolution of 20 000 at *m/z* 200 (full width at half maximum [FWHM]) and mass error within 0.5 milliDalton (mDa) were obtained.

Operating conditions

Separation was completed using a reverse phase step gradient elution scheme from 90% H_2O (0.1% formic acid with 0.01% ammonium hydroxide) to 100% acetonitrile (0.1% formic acid). The column temperature was kept constant at 20 °C and the flow rate was set at 0.3 millilitre/minute (ml/min) for the entire run giving a total run time of 5 min and gradient run time of 2.4 min. Injection volumes were set at 5 µL. A Kinetex® 1.7 µm EVO C18 100 Å (2.1 mm ID x 100 mm length) column was used (Table I).

Table I: Step gradient elution scheme for the UHPLC method

Time (min)	Flow rate (ml/min)	% H_2O (0.1% formic acid, 0.01% NH_4OH)	% ACN (0.1% formic acid)
0 (initial)	0.3	90	10
0.1	0.3	90	10
2.5	0.3	0	100
3	0.3	0	100
3.1	0.3	90	10
5	0.3	90	10

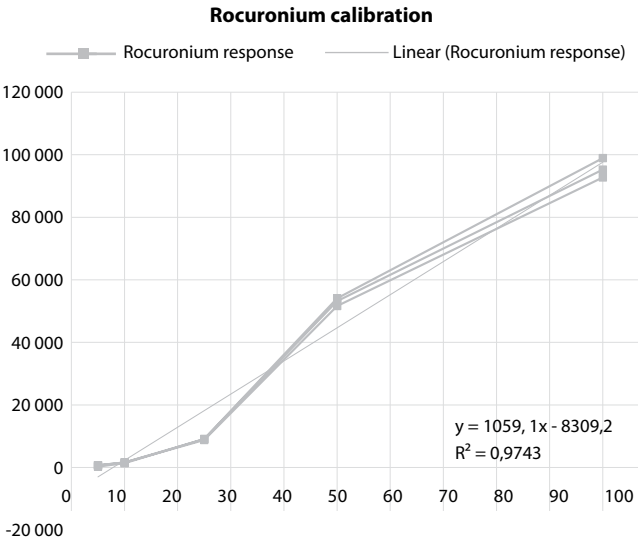
All analyses were conducted using positive mode ionisaton giving $[M+H]^+$ molecular ion adducts. The source conditions were as follows: the capillary voltage for electrospray ionisation (ESI) was 2.6 kV for positive mode ionisation. The source temperature was set at 120 °C, the sampling cone voltage at 25 V, extraction cone voltage at 4.0 V and cone gas (nitrogen) flow at 10.0 L/Hr. The desolvation temperature was set at 350 °C with a gas (nitrogen) flow of 600.0 L/Hr.

Quantitative data-independent acquisition (DIA) was done using two simultaneous acquisition functions with low and high collision energy (MS^E approach) with a QTOF instrument. The high energy MS scan can be time aligned with the low energy scan in order to predict which fragment ions belong to which precursor ions, consequently the full mass spectrum is acquired. Fragmentation patterns can thus be used for qualitative confirmation. Fragmentation was performed using high energy collision induced dissociation (CID). The fragmentation energy was set at 2 V and 3 V for the trap and collision energy, respectively. The ramping was set from 3 to 4 V and 20 to 40 V for the trap and transfer collision energy, respectively. Mass spectral scans were collected every 0.3 seconds. The raw data was collected in the form of a continuous profile. Mass to charge ratios (*m/z*) between 50 and 1 200 Da were recorded.

Calibration

For calibration of the study design and method of the UHPLC-QTOF-MS analysis, a new specimen with the lowest time lapse between manufacture and testing was used. A specimen from LOT R015494 (expiry date January 2021) was retrieved and as per the standardised sampling and preparation model; the following concentrations were prepared: (Graph 1)

1. 100 µg/ml (100 parts per million [ppm])
2. 50 µg/ml (50 ppm)
3. 25 µg/ml (25 ppm)
4. 10 µg/ml (10 ppm)
5. 5 µg/ml (5 ppm)



Graph 1: Rocuronium bromide calibration

The quantification ion 529.4002 *m/z* ($[M]^+$ salt counterion) at retention time 1.05 minutes was used for all calibrations (Figure 2). The documented quan ion mass is 529.4002 m/z [mass error: 0.3mDa].

Statistical analysis

Analysis of data was done using Wilcoxon–Mann–Whitney rank-sum testing. Comparisons were performed between the different batches, the control groups (at various time intervals) and the experimental groups, including an additional analysis of an air-exposed, using STRATA 15.0®, (Statistics/Data Analysis, StrataCorp, Texas USA). The number of available samples was limited to 10 vials per carton; this translated to a limit of 20 vials to analyse over the study duration.

Results

Batch comparison analysis

Statistical analyses of the two batches were compared to establish if their data could be pooled to yield four data points of analysis per experimental group and control group, instead of two. The analysis revealed a *p*-value of 0.344, indicating no statistical difference between the two batches. The specimens were found to be comparable, with their documented three-month lifespan difference having no influence on concentration, thus allowing the two batches to be pooled for the duration of the study and enlarging the data pool for analysis.

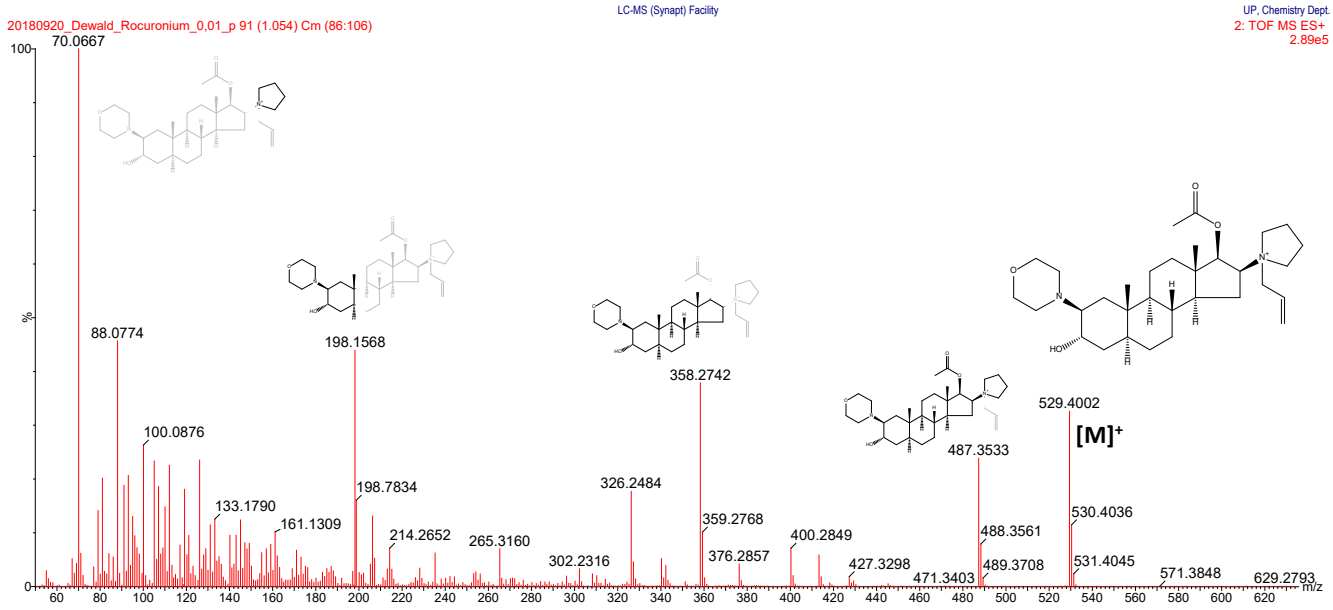


Figure 2: High energy mass spectrum of rocuronium (529.4002 m/z) ([M]$^+$ salt counterion) giving the MS/MS fragmentation pattern of the compound for qualitative confirmation

Condition specific comparison and analysis

The rocuronium specimens were subjected to different testing conditions, yielding the following results.

Single exposure

The 18 °C exposure limb of this group yielded a pre-exposure ppm value of between 8.21–8.66 compared to 8.31–8.36 for post temperature exposures. Whereas the 24 °C limb revealed a ppm range of 8.39–8.52 prior to temperature exposure and ppm range of 8.50–8.62 after exposure (Table II).

Double exposure

Because the manufacturer warns that the compound is only stable for a period of < 24 hours after breaking the air-tight seal, analyses were performed prior to the second exposure and then after the second cold-chain re-introduction only. The assumption was that the first exposure of this group would yield similar results to its corresponding single exposure group dataset.

In the 18 °C experimental limb, ppm values post first exposure, but pre second exposure ranged between 9.36–10.86 ppm; compared to 8.76–9.69 ppm post second exposure. Whereas the 24 °C limb showed a range of 8.74–9.44 ppm pre-secondary exposure and range of 9.86–11.06 ppm post secondary exposure (Table II).

Control groups

The next analysis compared the two control groups to each other at their respective interval testing. The first interval analysis

Table II: Data analysis from all groups

		Before exposure ppm		After exposure ppm		*p*-value	Air mod ppm	*p*-value
18 degrees single exposure	Batch A	8.21	8.31	8.32	8.31	0.273		
18 degrees single exposure	Batch B	8.56	8.66	8.39	8.36			
24 degrees single exposure	Batch A	8.47	8.52	8.62	8.61	0.067		
24 degrees single exposure	Batch B	8.39	8.43	8.52	8.50			
18 degrees double exposure	Batch A	10.86	10.31	8.76	8.81	0.465	7.89	0.02
18 degrees double exposure	Batch B	9.65	9.36	9.67	9.69		7.88	
24 degrees double exposure	Batch A	9.44	9.23	9.86	9.90	0.068	7.86	0.02
24 degrees double exposure	Batch B	8.74	8.55	11.01	11.06		7.86	

		Week 1		Week 6		Week 12		Week 1 vs Week 12
Control group 1	Batch A	8.41	8.45	7.87	7.89	8.11	8.33	*p*-value 0.068
Control group 1	Batch B	8.61	8.63	7.87	7.89	8.22	8.40	
Control group 2	Batch A	8.46	8.45	7.89	7.91	8.40	8.56	*p*-value 0.144
Control group 2	Batch B	8.40	8.37	7.89	7.90	8.53	8.62	
		p-value 0.149		*p*-value 0.248		*p*-value 0.043		
Global *p*-value experimental groups vs control group 1 week 1: 0.395								

was performed at week 1, followed by week 6, and week 12 (Table II). No statistically significant differences were found between the control groups for weeks 1 and 6; however, a statistically significant result was found at week 12.

No statistically significant intra-group differences in rocuronium were found when comparing week 1 to week 12 for either of the control groups (Table II).

On further analysis, comparing the rocuronium of the different experimental groups to Control group 1, a global *p*-value of 0.395 was obtained, indicating no statistical significance between any of the experimental groups and this refrigerated cold-chain control.

The final analysis introduced a secondary modifier. Experimental groups A2 and B2 that were reintroduced to the cold chain twice were used as a secondary control group; these specimens were exposed to air during their second 18 °C or 24 °C water bath exposures. This analysis provided a comparative data set as reference to ensure that the effect of accidental air entrainment was not overlooked and to verify if the manufacturer's recommendation regarding the airtight seal is of real significance.

When performing statistical analysis of this new experimental group, comparing it to the corresponding experimental group 2 data, a *p*-value of 0.020 was found. This indicated a clear statistically significant change when the specimens were exposed to air, irrespective of temperature change, with a mean decrease in ppm measure of 20% after a single air exposure.

Temperature data ranges

Prior to separation into the respective control groups, both recorders were placed in the same container recording the cold-chain during transport. This was done for inter-device comparison and calibration.

Thereafter, 10 minute interval temperature analysis spanning the 12 week period showed that the room temperature reached a maximum temperature of 27.5 °C and median of 24.9 °C, whereas the cold-chain temperatures reached a maximum temperature of 8 °C and minimum of 3.8 °C.

Discussion

The aim of this study was twofold: firstly, to see whether or not repeated cold-chain breakages and re-exposure back into the cold-chain would cause alterations in rocuronium's molecular structure, and secondly, to investigate the legitimacy of the manufacturer claims of product stability when rocuronium is deliberately removed from the cold-chain and stored at room temperatures for 12 weeks. This scenario could provide an alternative pragmatic storage solution if re-introduction of rocuronium into the cold-chain proved problematic.

Initially, two batches with different expiry dates were compared, and were shown to not differ significantly. Therefore, the data sets were combined in order to further strengthen subsequent statistical analyses.

The experimental groups were re-introduced into the cold-chain, either once or twice, after being exposed to either 18 °C or 24 °C temperatures. Importantly, there were no significant differences in any of the four experimental groups when comparing their pre-and post-exposure ppm measurements. There were also no significant differences between the post-exposure ppm measurements of the experimental groups compared to the maintained cold-chain Control group 1, which is deemed the ideal storage condition of rocuronium. This contradicts the manufacturer product insert that advises against cold-chain re-introduction practices. It is noted, however, that the sample exposure durations and quantity in the experimental setup was tightly controlled and monitored, which possibly does not reflect current clinical practice.

We found no statistically significant differences in the rocuronium ppm values between the refrigerated control group 1 and the shelf-stored control group 2 for the weeks 1 and 6 analysis. However, at week 12 the two groups showed a statistically significant result derived from a 0.263 ppm average difference between the two control groups. This translates to a 26 µg/ml difference in the clinical dosages. This is clinically insignificant. This confirms the manufacturer's assertion that the product may be kept at room temperature for at least 12 weeks without degradation. Interestingly, we could not demonstrate any significant time-based degradation based on age of the product over the twelve-week period.

The final analysis determining the effect of exposing the product to air provided significant results. Exposure to air resulted in a 20% reduction in ppm rocuronium within 24 hours. Care should be taken even if small quantities of the drug are aspirated for top-up dosing. In paediatric patients where rocuronium is drawn up and diluted appropriately in a syringe for repeated use during surgery also implies air contact and therefore, the effect of rocuronium might be altered especially during prolonged cases. This is in complete agreement with the manufacturer recommendation. During this trial, aspiration from each vial occurred on two occasions. However, as noted in the methodology, the specific technique of aspiration paid meticulous attention to preventing air contamination, which might not be reflective of current clinical practice.

Conclusion

In conclusion, this study found that up to two breakages in the cold-chain with subsequent exposure to clinically applicable increased temperatures had no statistical effect on the molecular structure of rocuronium bromide. Furthermore, this study also confirmed that rocuronium bromide may be stored at room temperature for up to 12 weeks without any significant deterioration. During clinical use of rocuronium bromide the detrimental effect of air exposure to the molecular integrity of this compound should be borne in mind especially during long cases with repeated top-up dosing or in cases where dilutions are done in advance.

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Conflict of interests

The authors declare no conflict of interest

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Ethical approval

Ethical approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria, study reference number 245/2018.

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