The Impact of Storage Conditions on the Stability of Clinical and Experimental Pharmaceuticals

by

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ABSTRACT

Administration of drugs should be based on a measured patient response wherever possible. To ensure a drug exhibits maximum efficacy and does not compromise patient acceptability or safety, it is essential to consider its physical, chemical, and biological stability outside of the body. Factors that can impact pharmaceutical stability include exposure to different environmental conditions (e.g. heat, light) and chemical processes (e.g. oxidation, hydrolysis) during transport, storage, and use. While pharmaceutical companies provide basic storage instructions, the impact of exposure to conditions that fluctuate from those recommended is not always evident. The aim of the investigations that form these doctoral studies was to determine the effect of different storage conditions, including solution pH and temperature, on the stability of a range of pharmaceuticals. These included clinically-used drugs, specifically remifentanil (an opioid analgesic), propofol (a short-acting anaesthetic), and tenecteplase (a thrombolytic agent), as well as an experimental plant extract still in development, EBC-46 (found to exhibit both anticancer and wound-healing properties). These compounds were chosen for investigation as they are of interest to industry partners and represent a diverse range of structures and pharmacological activity covering central nervous system, cardiovascular, and chemotherapeutic applications. To determine drug stability, a range of analytical techniques, including high-performance liquid chromatography (HPLC), mass spectrometry (MS), and UV spectrometry, were employed. The findings of the studies demonstrate that even minor changes in the storage environment can have a substantial impact on the pharmaceutical stability of these diverse agents, therefore controlling this environment is crucial in ensuring full potency is retained long term. Specifically, Study A shows that solution pH has the greatest influence on remifentanil stability while propofol degradation is affected by diluent electrolyte concentration, Study B shows that high temperature alters the clinical functionality of lyophilised tenecteplase, and Study C shows that temperature, solution pH, and light affect the stability of the EBC-46 pure product. The outcomes highlight to clinicians the importance of correct pharmaceutical storage and the impact it can have on the dose (and, therefore, efficacy) of the product that is administered to patients, and in some instances may inform best practice for our industry partners moving forward. Further research in this area has the potential to not only uncover methods of improving pharmaceutical stability in storage, but also optimising the quality of care provided to patients.

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RHD Thesis Declaration

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION

Paper 1: "The effect of concentration, reconstitution solution and pH on the stability of a remifentanil hydrochloride and propofol admixture for simultaneous co-infusion"

Status: Published in *BMC Anesthesiology*

Nature of Candidate's Contribution: Project administration, methodology, validation, investigation, formal analysis, writing (original draft), visualisation.

Nature of Co-Authors' Contributions: RV – project administration, methodology, validation, writing (reviewing and editing), visualisation. KB – conceptualisation, resources, writing (reviewing and editing). DA – conceptualisation, writing (reviewing and editing). PK – conceptualisation, writing (reviewing and editing), visualisation. AF – conceptualisation, supervision, writing (reviewing and editing).

Paper 2: "The effect of high storage temperature on the stability and efficacy of lyophilized tenecteplase"

Status: Published in *Prehospital and Disaster Medicine*

Nature of Candidate's Contribution: Project administration, methodology, validation, investigation, formal analysis, writing (original draft), visualisation.

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Paper 3: "Temperatures reached inside an emergency medical vehicle and paramedic response kit during an Australian summer"

Status: Paper to be submitted

Nature of Candidate's Contribution: Project administration, methodology, validation, investigation, formal analysis, writing (original draft), visualisation.

Nature of Co-Authors' Contributions: RV – project administration, conceptualisation, methodology, writing (reviewing and editing), visualisation. AF – conceptualisation, writing (reviewing and editing).

Paper 4: "The influence of temperature, pH, air and light exposure, and cellular enzymatic degradation on the novel antitumor compound EBC-46"

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Nature of Co-Authors' Contributions: RV – writing (reviewing and editing). AF – project administration, conceptualisation, visualisation, writing (reviewing and editing).

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Chapter 3: "Temperatures reached inside an emergency medical vehicle and paramedic response kit during an Australian summer" (article to be submitted)

Chapter 3: "The effect of high storage temperature on the stability and efficacy of lyophilized tenecteplase" (article published in *Prehospital and Disaster Medicine*)

Chapter 4: "The influence of temperature, pH, air and light exposure, and cellular enzymatic degradation on the novel antitumor compound EBC-46" (article to be submitted)

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LIST OF ABBREVIATIONS

AMI	acute myocardial infarction
ANOVA	analysis of variance
CI	confidence interval
DAD	diode array detector/detection
DTT	dithiothreitol
DVT	deep vein thrombosis
ECG	electrocardiogram
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ESV	emergency service vehicle
FBS	fetal bovine serum
FEU	Fibrinogen Equivalent Units
GABA	gamma-aminobutyric acid
GC	gas chromatography
HPLC	high performance liquid chromatography
ICU	intensive care unit
IV	intravenous
LC-MS	liquid chromatography-mass spectrometry
mAb	monoclonal antibodies
MI	myocardial infarction
MKT	mean kinetic temperature
MS	mass spectrometry
PAI	plasminogen activator inhibitor
PE	pulmonary embolism
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PRK	paramedic response kit
QAS	Queensland Ambulance Service
QIMR	Queensland Institute of Medical Research
RPM	revolutions per minute
RT	room temperature
rt-PA	recombinant tissue plasminogen activator
SD	standard deviation
SDS	sodium dodecyl sulfate

SEC	size-exclusion chromatography
SEM	standard error of the mean
STEMI	ST-elevation myocardial infarction
t-PA	tissue plasminogen activator
USP	United States Pharmacopeia
UV	ultraviolet

LIST OF RELEVANT PUBLICATIONS

1. "The effect of concentration, reconstitution solution and pH on the stability of a remifentanil hydrochloride and propofol admixture for simultaneous co-infusion"
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     Henkel, E, Vella, R, Behan, K, Austin, D, Kruger, P & Fenning, A 2020, 'The effect of concentration, reconstitution solution and pH on the stability of a remifentanil hydrochloride and propofol admixture for simultaneous co-infusion', *BMC Anesthesiology*, vol. 20, article no. 283, pp. 1-12.

2. "The effect of high storage temperature on the stability and efficacy of lyophilized tenecteplase"
   Emily Henkel, Rebecca Vella, Andrew Fenning
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     Henkel, E, Vella, R & Fenning, A 2020, 'The effect of high storage temperature on the stability and efficacy of lyophilized tenecteplase', *Prehospital and Disaster Medicine*, vol. 35, no. 5, pp. 501-507.

1. INTRODUCTION

Administration of drugs should be based on a measured patient response wherever possible (Roberts & Freshwater-Turner 2007). Two aspects of a drug's pharmacology that affect this are its pharmacokinetics (the drug concentration-time courses in body fluids resulting from administration of a certain drug dose) and pharmacodynamics (the observed effect resulting from a certain drug concentration) (Meibohm & Derendorf 1997). To ensure a drug exhibits maximum efficacy and safety when it is administered to patients, it is essential to consider its physical, chemical and biological stability outside of the body. Drug stability can be defined as "the extent to which a product retains, within specified limits and throughout its period of storage and use (i.e., its shelf life), the same properties and characteristics that it possessed at the time of its manufacture." (Kommanaboyina & Rhodes 1999). Degradation can depend on conditions such as concentration, pH, and temperature, and may involve chemical decomposition of the active ingredient/s or excipients, or physical changes to the dosage form itself. Factors that can impact pharmaceutical degradation include: the stability of the active ingredient; interactions between components, excipients and/or diluents; and exposure to different environmental (e.g. heat, light) and chemical (e.g. oxidation, hydrolysis) conditions during transport, storage and use (Kommanaboyina & Rhodes 1999; Gbenga & Taiwo 2015). If drug degradation were to unknowingly occur, patient safety could be compromised through several adverse effects including active-drug deterioration, altered biological activity, decreased solution homogeneity, and formation of toxic by-products (Kommanaboyina & Rhodes 1999).

While pharmaceutical companies provide basic storage instructions, the impact of exposure to conditions that fluctuate from those recommended, e.g. extreme temperature or pH, is not always evident for each specific medication. Furthermore, there are circumstances during transport, storage, and use where the external conditions cannot always be controlled. Further research in this area has the possibility to not only uncover methods of improving pharmaceutical stability in storage, but also optimising the quality of care provided to patients.

The investigations that form these doctoral studies examined the effect of different storage conditions, including temperature fluctuations, drug concentration, and the pH of the solution, on the stability of both clinically-used drugs (remifentanil, propofol, and tenecteplase) and an experimental drug still in development (EBC-46). Furthermore, each project was developed as part of a collaboration with industry partners. Remifentanil, an opioid analgesic, and

propofol, a short-acting anaesthetic, are commonly used during surgery and for managing pain in intensive care unit (ICU) patients. This study was established in collaboration with clinicians at Rockhampton Hospital who were interested in improving the efficiency of remifentanil and propofol administration through admixing. Tenecteplase is a thrombolytic agent used by paramedics to manage blood clot diseases such as acute myocardial infarction (AMI) and pulmonary embolism (PE). Emergency service personnel have found administration of tenecteplase to have inconsistent results, with concerns that vehicle storage, specifically high temperatures, may be affecting the efficacy of the drug (2017, pers. comm.). Finally, EBC-46 is an investigational drug currently undergoing human trials that has been found to exhibit both anticancer and wound-healing properties. Researchers at the Queensland Institute of Medical Research (QIMR Berghofer) wanted to perform preliminary stability studies on EBC-46 while it was still in the early phases of drug development.

All investigations utilised a range of analytical techniques, including high performance liquid chromatography (HPLC) and mass spectrometry (MS), to determine drug concentration and stability. The results obtained will enlighten clinicians and/or researchers on how storing pharmaceuticals can impact the dose, and therefore efficacy, that is administered to patients.

1.1. STUDY A – The stability of remifentanil and propofol in combination

1.1.1. Remifentanil

Remifentanil is a synthetic opioid analgesic that selectively binds μ opioid receptors. Unlike other opioid compounds, remifentanil is not metabolised by the liver but is subjected to widespread chemical hydrolysis catalysed by non-specific esterases circulating in blood and tissue (Haider et al. 1996; Navapurkar et al. 1998; Said et al. 2011). This makes it suitable to administer to patients who have some degree of organ dysfunction, such as liver disease or renal impairment, that are commonly found in the ICU (Dershwitz et al. 1996; Pitsiu et al. 2004). Hydrolysis at the methyl ester located on the *N*-acyl moiety (Figure 1.1) occurs within minutes, making remifentanil an ultra-short acting opioid (onset of action of approximately one minute) with a brief recovery time for patients (Davis et al. 2002; Pitsiu et al. 2004). Compared with other opioids, remifentanil has been shown to have a shorter duration of mechanical ventilation and quicker discharge from ICU, possibly offsetting the increased cost associated with this drug by improving patient recovery (Battershill & Keating 2006).

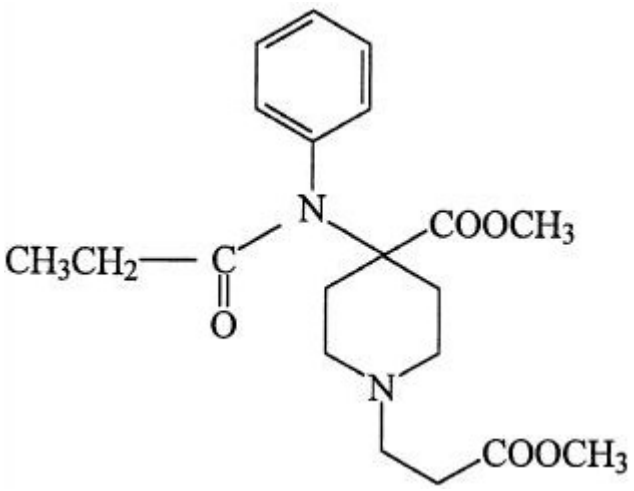

Figure 1.1. Structure of remifentanil.
Source: Vishwanathan & Stewart 1999

The short half-life of 2 – 3 minutes reduces unwanted effects, including postoperative respiratory suppression and accumulation (Zhang et al. 2009). Furthermore, the primary, less-potent carboxylic acid derivative produced from the hydrolysis process, remifentanil acid, is almost completely inactive and secreted in urine (Brady et al. 2005; Bossù et al. 2006). Due to this elimination process, the metabolite accumulates in patients with severe renal impairment, however the potency of remifentanil acid is 1/4600 that of its parent compound and has not been associated with prolonged effects at the μ-opioid receptor (Hoke et al. 1997; Pitsiu et al. 2004). As such, it is commonly used in conjunction with general anaesthesia during surgery (Stewart et al. 2000). One of the most common anaesthetic drugs often coupled with remifentanil is propofol.

1.1.2. Propofol

Propofol (Figure 1.2) is a short-acting general anaesthetic with rapid onset (approximately 30 seconds) and recovery. The mechanism of action, like other general anaesthetics, is not fully understood (Propofol Sandoz Product Information 2009). However, it is believed that propofol positively modulates the inhibitory function of the gamma-aminobutyric acid (GABA) neurotransmitter through $GABA_A$ receptors (Trapani et al. 2000). Compared with another short-acting sedative, midazolam, propofol was shown to be more effective in quality of sedation and shortening of time between termination of sedation and extubation (Rowe & Fletcher 2008).

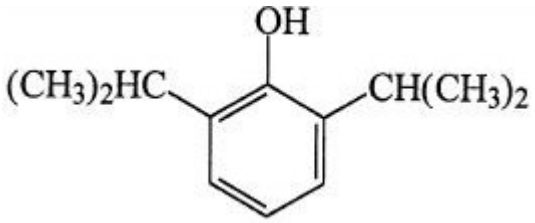

Figure 1.2. Structure of propofol.
Source: Vishwanathan & Stewart 1999

Following administration, the metabolism of propofol primarily occurs in the liver through oxidation and conjugation reactions via cytochrome P-450 enzymes that are essential for drug metabolism (Favetta et al. 2002; Lynch & Price 2007). The major metabolites are quinols, such as 2,6-diisopropyl-1,4-quinol (added hydroxyl group in the propofol benzene ring) and glucuronide products (glucuronide transferred to the hydroxyl group of propofol), including a glucuronic acid conjugate of propofol and the glucuronic acid and sulphate conjugates of the quinol hydroxylated derivative (Simons et al. 1988; Vree et al. 1999; Palmer 2007; Propofol Sandoz Product Information 2009). These resultant metabolites are physiologically inactive, having no beneficial effects on the sedating ability of propofol, and are excreted by the kidneys; 88% is recovered in urine, with less than 0.3% unchanged (Simons et al. 1988; Cussonneau et al. 2007; Propofol Sandoz Product Information 2009). Long-term infusion of propofol may lead to propofol infusion syndrome, resulting in severe metabolic acidosis, muscle necrosis, and arrhythmias, making it unsuitable for use in children under three years of age (Rowe & Fletcher 2008). Thus, patients must be closely monitored when administered propofol, particularly for the arrhythmogenic and serum triglyceridemia side effects (Laguay et al. 2010).

1.1.3. Remifentanil and propofol

Both remifentanil and propofol have a rapid equilibration between blood and the brain, and therefore rapid onset of action (Propofol Sandoz Product Information 2009; Ultiva (remifentanil hydrochloride) for Injection Product Information 2011). Both compounds also have a short duration of action due to rapid distribution and clearance, with their effects directly correlating with plasma concentration (Propofol Sandoz Product Information 2009; Ultiva (remifentanil hydrochloride) for Injection Product Information 2011). These attributes allow for ideal control over pain and anaesthesia management and afford faster recovery for patients as the anaesthetic effects resolve quickly. Remifentanil and propofol interact synergistically when

co-administered; propofol reduces remifentanil requirements for suppression of responses to different stimulation prior to surgery, while remifentanil decreases propofol concentration when returning a patient to consciousness (Mertens et al. 2003). Propofol has also been found to inhibit the metabolism of opioid analgesics, resulting in their increased plasma concentrations (Vuyk 1997). Other advantages of using a combination of propofol and remifentanil include: it is both cost effective and easy to use (Mertens et al. 2003; Brady et al. 2005); would only require one inlet on the catheter system, meaning efficient medication administration for patients requiring several continuous infusions via invasive ventilation; and remifentanil inhibits bacterial growth within the propofol lipid emulsion (Apan, Apan, Şahin and Çakırca, 2007; Erden et al. 2013). These benefits have led to this combination being used as effective moderate sedation for procedures such as colonoscopies, eye surgery, and fiberoptic bronchoscopy, while a current clinical trial is exploring its use in breast cancer surgery (Holas et al. 1999; Berkenbosch et al. 2004; Mandel et al. 2008; U.S. National Library of Medicine 2019).

A method used to ensure patients are receiving the correct dose of pharmaceuticals is based on Dixon's up-and-down method (Dixon 1965). This involves administering a starting dose of remifentanil to patients that is the minimum amount expected to result in a positive response, and if the response is not sufficient the dose is increased by a predetermined volume (Kumar & Tyagi 2009). This increase in concentration continues until the desired response is achieved (Hayes et al. 2008; Demirkaya et al. 2012). According to the Ultiva (remifentanil hydrochloride) for Injection Product Information guide (2011), the adult dosing guidelines for injectable remifentanil delivered as a slow bolus dose is 1 μg/kg over 60 seconds for induction of anaesthesia, and 0.5 – 1 μg/kg for maintenance of anaesthesia with nitrous oxide, isoflurane or propofol. For continuous infusion, the starting rate is 0.5 – 1 μg/kg/min for induction of anaesthesia, or 0.25 μg/kg/min for maintenance of anaesthesia with propofol with a range of 0.05 – 2 μg/kg/min (Ultiva (remifentanil hydrochloride) for Injection Product Information 2011).

1.1.4. Remifentanil/propofol stability

It is crucial that the compatibility of any drugs being considered for admixture be established before patient administration commences. If degradation unknowingly occurs, the drugs may produce an insufficient anaesthetic and/or analgesic response that necessitates a dose increase without knowledge of the concentration that has already been administered to the patient. Determining the stability of a remifentanil-propofol mixture, and finding ways of

improving it, will not only ensure its safety and efficacy when used in patients, but may also provide avenues for the use of other combinations of opiate agonists and short-acting anaesthetics in a clinical setting.

Remifentanil and propofol mixtures have already been trialled for several procedures as their use offers a number of advantages for those in remote or busy medical facilities, including increased ease-of-use (as only one syringe is required to inject both drugs) and enhanced patient recovery times (Brady et al. 2005; Bedocs, Evers & Buckenmaier III 2019). However, this is not typical practice; manufacturers of remifentanil advise against mixing with propofol, with no further explanation provided (Ultiva (remifentanil hydrochloride) for Injection Product Information 2011). While this may simply be due to the opacity of propofol making it difficult to detect any precipitation that occurs following mixing of the drugs, the rationale could be more complex; factors that can affect the stability of drugs include the storage vessels used, the concentration of drug added, the diluent used for reconstitution, the resulting pH of the mixture, and adverse drug interactions. Interestingly, manufacturers of Ultiva, Glaxo-Wellcome Inc., supported a study by Stewart et al. in 2000 that investigated the stability of remifentanil hydrochloride and propofol mixtures in polypropylene syringes and polyvinylchloride bags over a period of 36 hours. High (50 µg/mL) and low (5 µg/mL) concentrations of remifentanil were added to 10 mg/mL propofol, with drug stability deemed unsuitable when 90% of the original concentration remained (Stewart et al. 2000). It was found that the high remifentanil concentration remained stable for longer and that the mixture had greater stability when stored in syringes, possibly suggesting some level of compatibility. Further to the Stewart et al. (2000) study, Gersonde, Eisund, Haake and Kunze (2017) mixed and stored propofol with other sedatives and analgesics, including remifentanil reconstituted with 0.9% saline solution, in a syringe for 7 days. The findings were similar to that of Stewart et al. (2000) in that the greatest change in drug concentration occurred in mixtures containing the least amount of remifentanil (Gersonde, Eisund, Haake & Kunze 2017). A pilot study performed at CQUniversity observed the effect of mixing a range of remifentanil concentrations (10 – 50 µg/mL) in a stable 10 mg/mL of propofol in glass over 24 hours, as well as the effect of reconstituting remifentanil in a 0.9% saline solution to decrease pH. The main conclusion of this investigation was that reconstituting remifentanil with 0.9% saline solution significantly improved remifentanil stability compared to water reconstitution (unpublished findings). As manufacturers do not include any rationale with their instructions, all these findings warrant additional exploration.

Most studies investigating this topic only include a few concentrations of remifentanil and/or propofol, and mixtures are stored in plastic syringes or bags (Stewart et al. 2000; Gersonde, Eisend, Haake & Kunze 2017). There are a lack of studies that determine the impact of mixing on both remifentanil and propofol concentration, and more in-depth knowledge is required on how a variety of initial remifentanil concentrations and solution pH values affect mixture stability when stored in glass. The pH is a particularly important factor as hydrolysis of the ester group of remifentanil readily occurs at a pH range of 7 – 7.5 (Stewart et al. 2000). Following reconstitution with water, remifentanil has a pH of 3.0 (Glass 1995). In comparison, propofol can have a pH ranging from 6 – 8.5 (Tan & Onsiong 1998). Thus, reconstitution with a solution that is of a more suitable pH may provide one method of stabilising remifentanil when mixed with propofol.

The aims of this investigation were to determine the effect of mixing the drugs on both remifentanil and propofol stability over time, along with the effect of remifentanil concentration, the diluent used, and the resulting pH on the stability of both drugs in isolation and in solution. This research will not only determine the compatibility of a remifentanil and propofol mixture from a chemical perspective, but also has the potential to uncover methods of storing the mixture that may improve its stability and, as a result, provide avenues for the use of other agonists and anaesthetics in combination.

1.1.5. Remifentanil/propofol analysis techniques

As propofol is an opaque liquid, using visual assessment alone to detect incompatibility is very difficult. Analytical techniques provide a way of assessing changes in drug concentration, and therefore stability, in real time. Analysis of both remifentanil and propofol has been achieved using gas chromatography (GC) (Grosse et al. 1994; Bjorksten, Chan & Crankshaw 2002; Miekisch et al. 2008), however, high-performance liquid chromatography (HPLC) is the technique more commonly employed as GC methods require time-consuming sample preparation and analytical procedures, and longer run times (Bossù et al. 2006). HPLC utilises high pressure to force solvent (the mobile phase) through a packed column containing very fine particles (the stationary phase), resulting in high-resolution separations based on polarity differences between the analyte and the stationary phase (Harris 2003; Agilent Technologies 2016). The system consists of a solvent delivery system, a sample injection valve, a high-pressure column, a detector, and a computer to control the system and display results (Harris 2003). A very basic schematic of a modern HPLC system can be seen in Figure 1.3. HPLC

analysis easily enables the quantification of remifentanil and propofol concentrations either in isolation or after mixing over time, so any changes in drug stability can be quickly identified.

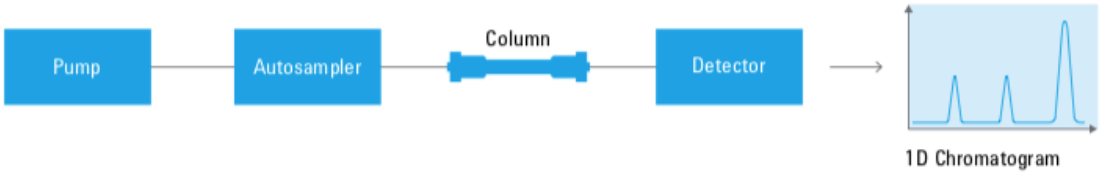

Figure 1.3. Basic illustration showing the components of a typical modern HPLC system. Source: Agilent Technologies 2016

Specifically, both drugs have been analysed using reversed-phase chromatography on a C18 column (Vree et al. 1999; Crespo et al. 2006; Li et al. 2006; Zhou et al. 2007; Said et al. 2011). Silica gel is a commonly used stationary phase within HPLC columns; reversed-phase chromatography indicates that the normally polar silanol groups on the surface of the silica gel have been chemically modified to be non-polar, resulting in increased reproducibility of retention times (Agilent Technologies 2016). Octadecylsilane, also known as octyldecylsilane or C18, indicates the number of alkyl chains attached to the silica surface and is one of the most common bonded phases used (Agilent Technologies 2016). Compared to other commonly-used columns (such as the C8 column), analysis on a C18 offers the general advantages of increased sensitivity (as the analytes are retained more strongly) and the ability to be used with a wide variety of compounds at significantly different pH values (Agilent Technologies, Inc. 2007). While fluorescence detection has been used for propofol analysis (Yeganeh & Ramzan 1997; Li et al. 2006; Cussonneau et al. 2007), a variable wavelength UV diode array detector (DAD) has been employed for the detection of both drugs (Pavan et al. 1992; Haidar et al. 1996; Vree et al. 1999). Furthermore, an injection volume of 10 µL has been used for remifentanil and propofol analysis (Vishwanathan & Stewart 1999).

To achieve maximum solute resolution, separate HPLC protocols are used to analyse a mixture of remifentanil and propofol. This involves detection at separate wavelengths, columns of different dimensions and with different flow rates, and the use of unique mobile phases. A suitable method produces peaks that are narrow, have a reasonable retention time, and have a symmetrical shape with no leading or tailing. Leading peaks occur when the concentration of solute increases and becomes more soluble in the stationary phase, and is

usually the result of introducing excess solute to the column. Tailing peaks arise when small quantities of solute are retained more strongly on the column (Harris 2003).

Column dimensions affect the resolution, sensitivity, solvent usage, efficiency and speed of the analysis (Agilent Technologies 2016). The mobile phase used affects selectivity and sample retention, and is likely dictated by sample solubility. Controlling mobile phase pH is also important for optimising selectivity, peak shape, retention, and column integrity (Agilent Technologies 2016). As important as the chemicals chosen to act as the mobile phase is the ratio at which they are mixed, as this can manipulate retention and peak shape. Figure 7.1 (see Chapter 7) demonstrates this using only minor alterations to the mobile phase ratio when analysing a propofol sample. The flow rate depends on the column type, and length and diameter used, as the interaction between the analyte and the column affects how fast the mixture can be effectively separated. Like the mobile phase ratio, the flow rate can be easily manipulated to achieve desired peak shape. An example of this can be seen in Figure 7.2 (see Chapter 7), where increasing the flow rate reduced the tailing of a propofol peak (Agilent Technologies 2016).

For degradation product determination, a combination of liquid chromatography and mass spectrometry (LC-MS) was used. Liquid chromatography coupled with mass spectrometry involves two separation techniques. First, liquid chromatography separates and detects the components based on their interaction with the mobile and stationary phase. These components then enter the ion source of the mass spectrometer and are converted from neutral molecules in liquid to charged ions in gas through ionisation. Finally, mass spectrometry separates the components further based on their mass-to-charge ratio (m/z) (Agilent Technologies 2016). The mass spectrum produced indicates the masses (and intensity) that are present at each chromatographic retention time, allowing peaks to be identified (Agilent Technologies 2016). It is, therefore, a useful technique for identifying breakdown/degradation products of known parent compounds.

1.1.5.1. Remifentanil analysis on HPLC

Remifentanil has been analysed on a column with dimensions of 150 x 4.6 mm, particle size of 5 µm, at a flow rate of 1.5 mL/min (Haidar et al. 1996). The mobile phase is an important consideration for remifentanil in particular, as ionisation occurs under reversed-phase conditions due to the presence of an amine functional group and a pKa value of 7.1 (Trivedi, Shaikh & Gwinnut n.d.). To alleviate this effect and produce more accurate results, a buffer

such as sodium dihydrogen phosphate (NaH_2PO_4), formic acid, or ammonium acetate (selected for this specific study) is commonly used (Haidar et al. 1996; Zhang et al. 2009; Said et al. 2011); this ensures degradation of remifentanil is only affected when mixed with propofol and not via interaction with the mobile phase. In addition to the buffer, the mobile phase for remifentanil analysis typically consists of a mixture of water and either methanol or acetonitrile (Crespo et al. 2006; Zhang et al. 2009; Said et al. 2011). For UV detection of remifentanil, a wavelength of 210 nm has not only been used in past studies (Haidar et al. 1996) but has previously been confirmed as the most suitable for remifentanil (see Figure 7.3 in Chapter 7).

1.1.5.2. Propofol analysis on HPLC

Column specifications vary for propofol analysis, with studies commonly using 250 x 4.6 mm, with pore size ranging from 5 – 10 µm (Vree et al. 1999; Pavan et al. 1992; Puri et al. 2012). The mobile phase for propofol analysis typically consists of a mixture of water and either methanol or acetonitrile (Yeganeh & Ramzan 1997; Li et al. 2006; Cussonneau et al. 2007). UV detection has been achieved at a wavelength of 270 nm (Pavan et al. 1992; Vree et al. 1999; Cussonneau et al. 2007) and was also previously confirmed as the most suitable for propofol (see Figure 7.3 in Chapter 7).

1.1.5.3. Remifentanil analysis on HPLC-MS/MS

Mass spectrometry has been shown to be appropriate for the detection of remifentanil following HPLC separation (Zhou et al. 2007; O'Connor et al. 2016). Further to this, numerous studies have coupled liquid chromatography with tandem mass spectrometry (HPLC-MS/MS), which utilises two mass filters in the same instrument (Bender et al. 1999; Cooreman et al. 2010; Said et al. 2011; Agilent Technologies 2016). This method has the benefits of low detection limits and analytical run times (Bossù et al. 2006). Studies suggest the most common practice for remifentanil detection is to run the MS in the positive ion mode and use nitrogen as the desolvation gas (Cooreman et al. 2010; Said et al. 2011; O'Connor et al. 2016).

1.2. STUDY B – The stability of lyophilised tenecteplase at high temperatures

1.2.1. Tenecteplase

Tenecteplase (marketed as TNKase) is a thrombolytic agent used in the management of diseases involving blood clots, such as acute myocardial infarction (AMI) (Semba et al. 2003;

Baruah et al. 2006). Thrombolytic therapy has been found to reduce the mortality rate in patients with suspected AMI, particularly those with ST elevation or bundle-branch block, regardless of age, gender, blood pressure, heart rate, or previous history of MI or diabetes. The magnitude of benefit for this therapy is also inversely correlated with time course of administration (Fibrinolytic Therapy Trialists' (FTT) Collaborative Group 1994).

Specifically, tenecteplase is classed as an intravenous direct recombinant tissue plasminogen activator (rt-PA) that elicits its effects by binding to fibrin in blood clots and converts plasminogen to the natural fibrinolytic agent plasmin via a single peptide bond cleavage (Figure 1.4) (Khan & Gowda 2003). This lyses the clot, as plasmin is a non-specific protease that breaks down the fibrinogen and fibrin contained within the clot (Baruah et al. 2006). It has been found that administration of 30, 40 or 50 mg of tenecteplase results in decreases in both circulating fibrinogen (4% - 15%) and plasminogen (11% - 24%) in a concentration-dependent manner (Baruah et al. 2006).

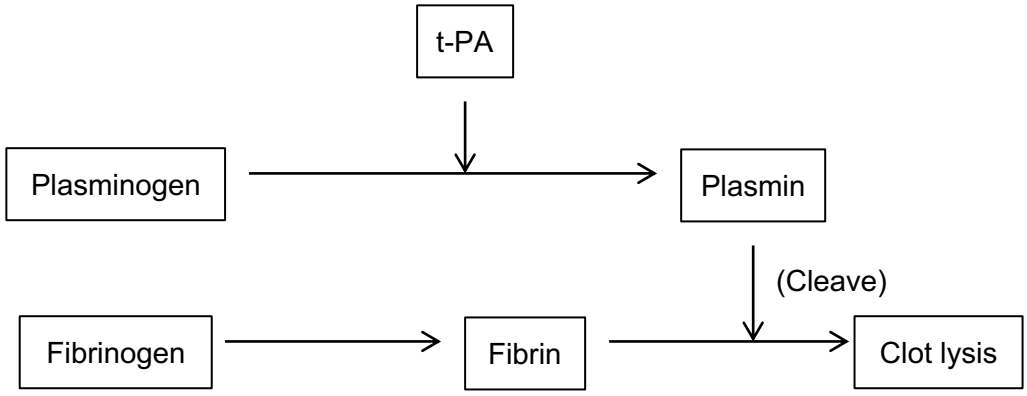

Figure 1.4. Tenecteplase mechanism of action. Platelet aggregation at the site of vascular injury is reinforced through the binding of the plasma protein fibrinogen, forming a platelet plug that is further bolstered by fibrin monomers generated by the enzyme thrombin. Tissue plasminogen activators such as tenecteplase convert inactive plasminogen to plasmin that is then able to break down fibrin within a clot, forming fibrin degradation products (Riley et al. 2016).

The development of tenecteplase was derived from the first generation thrombolytic, alteplase; it is a bioengineered triple-combination variant established to overcome limitations of previous fibrinolytic therapies (Assessment of the Safety and Efficacy of a New Thrombolytic (ASSENT-2) Investigators 1999). The structure of tenecteplase is similar to that of other proteins and characterised by five regions: a fibronectin finger, an epidermal growth

factor-like module (EGF), two kringle modules (mediates interaction with other proteins), and a serine protease domain (responsible for the enzymatic conversion of plasminogen to plasmin) (Figure 1.5) (Novokhatny, Ingham & Medved 1991; Kliche et al. 2014). The single-chain amino acid sequence that comprises tenecteplase has been altered at the T, N, and K domains of the molecule (Table 1.1) (Semba, Sugimoto & Razavi 2001). Tenecteplase exhibits clot lysis activity in both its one- and two-chain forms, the latter obtained through cleavage between amino acids 275 and 276 on contact with plasmin (Jiang et al. 2010; Kliche et al. 2014). The one-chain form has enhanced fibrin specificity that improves its ability to dissolve older clots and reduces non-cerebral bleeding difficulties, while cleavage to the two-chain form induces full thrombolytic activity (Loscalzo 1988; Assessment of the Safety and Efficacy of a New Thrombolytic (ASSENT-2) Investigators 1999; Kliche et al. 2014). Like naturally occurring t-PA, tenecteplase exhibits two glycoforms; type I contains three carbohydrate structures at asparagine residues 103, 184 and 448 (termed glycosylation sites 1, 2 and 3 and located in the kringle 1, kringle 2, and protease domain, respectively), while type II lacks the carbohydrate at asparagine 184 (Kliche et al. 2014).

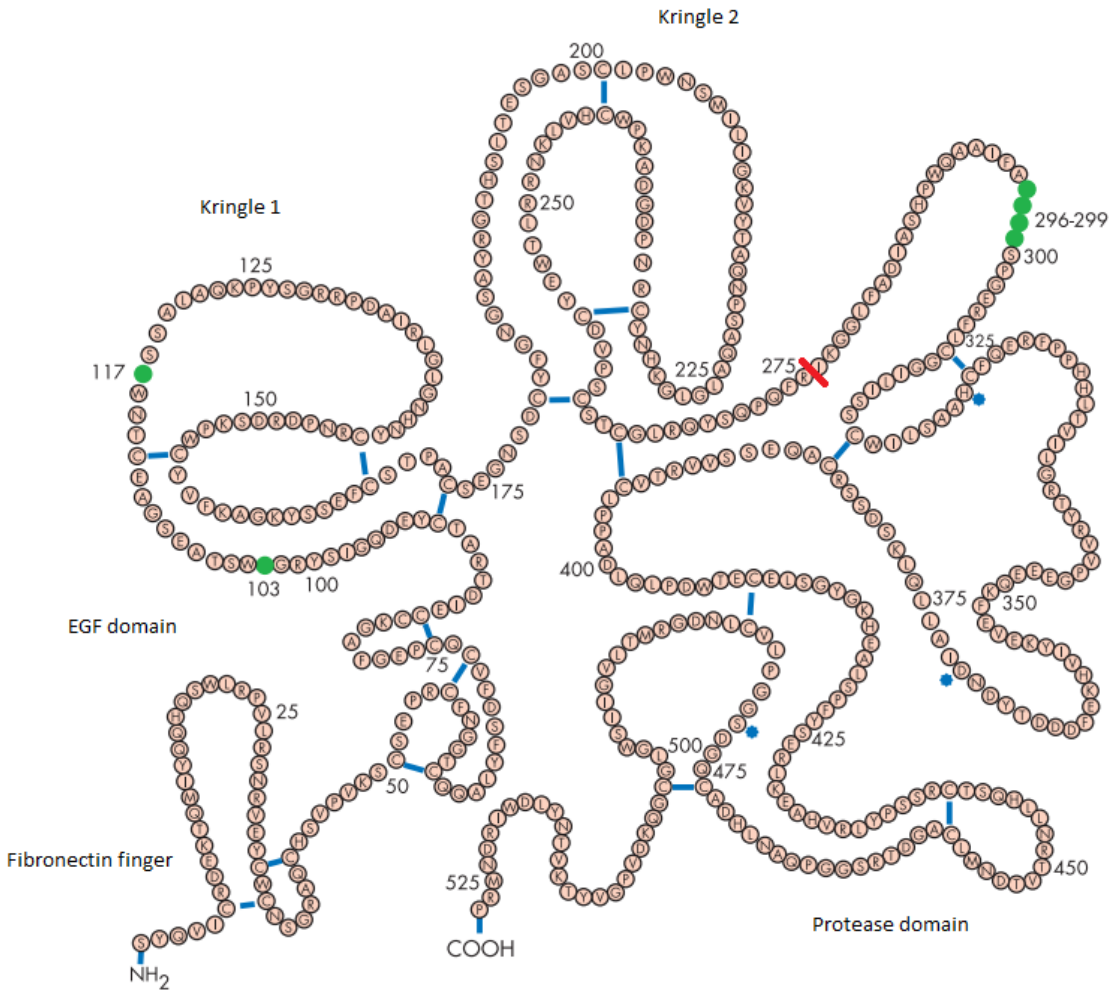

Figure 1.5. Structure of tenecteplase showing domains, disulfides and the plasmin cleavage site (Arg 275) for conversion from single-chain to two-chain (Benedict et al. 1995). The blue dots represent the location of active site residues, while the green dots represent where amino acid modification has occurred from alteplase.

Source: figure adapted from Nordt & Bode 2003.

Although more expensive than alteplase, tenecteplase exhibits four-times slower plasma clearance (from removal of the carbohydrate side chain that facilitates hepatic elimination at position 117), has 80-fold greater resistance to plasminogen activator inhibitor (PAI-1), has increased bioavailability, is not immunogenic, and exhibits a longer half-life (20-24 minutes compared to 4 minutes) (Assessment of the Safety and Efficacy of a New Thrombolytic (ASSENT-2) Investigators 1999; Nordt & Bode 2003; Baruah et al. 2006). This longer half-life is a result of the modification at position 103 that leads to a new glycosylation site, where a carbohydrate chain links and enlarges the molecule (Nordt & Bode 2003). Because of prolonged plasma half-life, tenecteplase can be administered as a single bolus (Baruah et al.

2006). Administration as a single bolus is simple and fast to perform compared with infusion, meaning the target clot is quickly exposed to a higher concentration of t-PA. This rapidly reduces the infarct size and increases preservation of left ventricular function (Benedict et al. 1995). Although this can cause increased bleeding related to plasmin generation, tenecteplase has 14-times greater fibrin specificity than alteplase as no domain is missing in the molecule, therefore allowing it to target the infarct-related clot while minimising systemic plasminogen activation and resulting in less fibrinogen depletion (Keyt et al. 1994; Nordt & Bode 2003; Baruah et al. 2006).

Table 1.1. Amino acid modification and associated benefits of tenecteplase

Alteplase	Tenecteplase	Advantage of modification
Thr^{103}	Asn	• Increased half-life • Resistance to plasminogen activator inhibitor (PAI-1) • Lysis of arterial thrombi
Lys^{296}-His-Arg-Arg	Ala-Ala-Ala-Ala	
Asn^{117}	Gln	• Slower clearance • Increased resistance to PAI-1

A major side effect of thrombolytic therapy is bleeding, the most serious being intracranial bleeding, and is a consequence of plasmin-mediated proteolysis. The main contributing mechanism is dissolution of fibrin in the haemostatic plug by plasmin, while other factors include the cleavage of fibrinogen, factor V, and factor VIII (producing a systemic hypocoagulable state), and platelet dysfunction due to plasmin cleaving platelet surface receptors (Levine et al. 1995).

1.2.2. Tenecteplase stability

Protein drug molecules such as tenecteplase have a structural complexity that makes them sensitive to degradation via numerous environmental conditions (Alsenaidy et al. 2014). As only minimal changes in structure can vastly affect the stability, efficacy and safety of a drug,

it is vital to maintain its chemical configuration during manufacturing, storage, shipment, and patient administration (Kommanaboyina & Rhodes 1999; Alsenaidy et al. 2014). Limiting thermal degradation in particular may be achieved by storing medications at controlled room temperature unless indicated otherwise (Stein 2008). However, ensuring these requirements are maintained for long-term storage, particularly in emergency vehicles servicing rural areas that experience extreme temperatures, can be a challenging task (Brown, Krumperman & Fullagar 2004). Although the temperature thresholds suggested by manufacturers are minimums, it is not fully known to what extent these can be exceeded for each type of medication carried by emergency vehicles (Brown, Krumperman & Fullagar 2004).

It is common for therapeutic proteins such as tenecteplase to undergo lyophilisation, a process of freeze-drying that forms a dried solid and slows degradation reactions such that the protein retains long-term stability at ambient temperatures (Carpenter, Pikal, Chang & Randolph, 1997). Manufacturers of tenecteplase, including Genentech Inc. (marketed as TNKase) and Boehringer Ingelheim (marketed as Metalyse), suggest storing the lyophilised powder at controlled room temperatures not exceeding 30°C, or under refrigeration at 2 - 8°C (Genentech, Inc. 2011; Metalyse (Tenecteplase) Product Information 2016). A number of studies have been conducted investigating the stability of freezing reconstituted thrombolytic agents (including tenecteplase) in order to decrease wastage (Semba et al. 2003). Wiernikowski et al. (2000) investigated if rt-PA remained stable for 22 weeks when frozen at -30°C. The concentration used was 1 mg/mL and samples were considered stable if they retained 95% of baseline potency. The results of this study found that rt-PA did remain stable when frozen for at least 22 weeks, with the expiry period of the drug extended to 4 months (Wiernikowski et al. 2000). A similar study by Semba et al. (2003) assessed the stability of small aliquots of tenecteplase when reconstituted and frozen at -20°C for one month, or when subjected to six freezing/thawing cycles and stored at -20 or -70°C. The final concentration used for both studies was 5 mg/mL. It was found that tenecteplase retained its baseline biological activity and biochemical properties compared to freshly reconstituted control samples for both studies (Semba et al. 2003). Ortega-Garcia & Blasco-Segura (2007) tested the stability of tenecteplase (following dilution with normal saline to a concentration of 25 µg/0.1 mL) when preloaded in syringes and frozen at -80°C for 388 days. Although the final assay revealed a tenecteplase recovery from the baseline concentration of 89%, all other assays indicated protein recovery that was within the United States Pharmacopeia (USP)

validity margins of 90 – 115%, indicating dilutions of tenecteplase can be frozen at -80°C for over one year (Ortega-Garcia & Blasco-Segura 2007).

In addition to freezing, the stability of reconstituted tenecteplase when stored at high temperatures has also been investigated. Lentz, Joyce and Lam (2011) looked at the stability and compatibility of reconstituted tenecteplase when stored at 37°C (to mimic body temperature) in glass vials, polysulfone/silicone central vascular access ports, and polyurethane or silicone catheters for 24 – 96 hours. There was no change in tenecteplase concentration found for all storage vessels (Lentz, Joyce & Lam 2011).

Potential microbiological growth in reconstituted tenecteplase necessitates its use immediately following preparation (Metalyse (Tenecteplase) Product Information 2016). Because of this, tenecteplase is transported and stored in its lyophilised form, placed within an insulated drug bag that is stowed in a designated space in emergency vehicles. As outlined previously, a number of researchers have performed stability studies on reconstituted tenecteplase; to our knowledge, there are no studies on the effect of storing non-reconstituted (i.e. lyophilised) tenecteplase at temperatures around or exceeding 30°C, possibly due to the assumption that products in their solid form are resistant to degradation. The need for this gap in knowledge to be addressed is of paramount importance because paramedics and other emergency personnel commonly use lyophilised tenecteplase to assist patients suffering from AMI during transportation to hospital, meaning it can be stored in vehicles for any period of time and, if unused, swapped between vehicles. This can result in the drug being subjected to numerous fluctuations in temperature, including temperatures exceeding those suggested by its manufacturers. In areas such as Queensland, temperatures inside a closed vehicle can rise from 36°C ambient temperature to 67°C within 15 minutes (King, Negus & Vance 1981). Although the effect of these temperatures on the stability of lyophilised tenecteplase is currently not fully understood, there are anecdotal accounts from Queensland Ambulance Service (QAS) paramedics that administering the correct dose to patients can produce a varied response, suggesting some degradation may be occurring unknowingly to varying extents. As such, this study aims to determine the impact of high temperatures on the stability and efficacy of non-reconstituted tenecteplase and provide suggestions on how to improve its stability when stored in emergency vehicles, thereby filling an important gap in knowledge.

1.2.3. Tenecteplase analysis techniques

Important factors to observe when determining the stability of proteins include colour/optical clarity, pH, protein concentration, protein monomer, single chain protein and *in vitro* bioactivity (Semba et al. 2003; Lentz, Joyce & Lam 2011).

1.2.3.1. Colour/optical clarity and pH

Change in colour or formation of precipitates is a good indication of physical or chemical degradation. Precipitation in particular is dangerous as it has the potential to cause catheter occlusion, embolism, adverse drug events, and therapeutic failure as a result of the reduction or elimination of the active drug (Grissinger 2016).

1.2.3.2. Concentration

When assessing the stability of a substance it is important to determine any decrease in concentration, as this can indicate that degradation has occurred. Liquid chromatography coupled with mass spectrometry (LC-MS/MS) has recently been used for the absolute quantification of protein drugs in biological matrices (Buscher et al. 2007). Specifically, HPLC has been utilised for protein concentration determination when assessing the stability of tenecteplase when frozen and stored (Semba et al. 2003). In terms of protein analysis, liquid chromatography is mainly used for qualitative purposes or relative protein quantification (comparison of protein levels between samples), where labelling techniques in combination with LC-MS/MS, such as isotope coded affinity tag (ICAT) labelling and per-methyl esterification of peptides, are usually required (Buscher et al. 2007).

Binding assays such as enzyme-linked immunosorbent assays (ELISA) are one of the most commonly used techniques for quantitative bioanalysis of proteins (Buscher et al. 2007). These require antibodies to specifically bind to the protein analyte, resulting in a response that is directly correlated to its concentration within the sample (Ji, Rodila & El-Shourbagy 2007). Benefits of utilising binding assays include they are sensitive, rapid, and low cost, however the preparation and purification of the specific antibodies is often difficult, while the determined protein concentration may only be a fraction of the total concentration of the analyte in the matrix (Ji, Rodila & El-Shourbagy 2007).

Although Buscher et al. (2007) suggested that LC-MS/MS could be an alternative to ELISA for the analysis of large proteins in very complex matrices, mass spectrometry is expensive

and therefore not always available. A simple, inexpensive method that has been employed for determining tenecteplase concentration is UV absorption via a spectrophotometer. This typically involves diluting tenecteplase samples in buffer, scanning the samples between wavelengths of 240 and 400 nm (with maximum absorption at 280 nm), and calculating protein concentration (Semba et al. 2003; Ortega-Garcia & Blasco-Segura 2007; Lentz, Joyce & Lam 2011).

1.2.3.3. Protein monomer

The protein monomer assay determines the amount of protein aggregation and fragmentation that has occurred and therefore gives an indication of protein breakdown (Semba et al. 2003). A loss in protein monomer corresponds to an increase in aggregates due to disruption of the protein's high order structure, resulting in a decrease in bioactivity (Lentz, Joyce & Lam 2011). Native size-exclusion chromatography (SEC) using a HPLC system has previously been used to detect tenecteplase protein monomer (Semba et al. 2003; Lentz, Joyce & Lam 2011). Unlike traditional HPLC, there is no interaction between sample compounds and column packing material in SEC. Instead, molecules diffuse into the pores of the packing material and are separated based on molecule size relative to pore size; therefore, larger molecules that are excluded from the pores elute first (Agilent Technologies 2016). This form of separation is mainly employed for polymer characterising/molecular weight determination, and for separating proteins (Agilent Technologies 2016).

1.2.3.4. Single chain protein

The single chain protein assay determines the amount of autolytic activity of the protease enzyme into single- and double-chain forms (Semba et al. 2003). A decrease in the one-chain form results in an increase in the two-chain form (Lentz, Joyce & Lam 2011). This is important to determine because although both forms show thrombolytic activity, the one-chain form has greater fibrin specificity due to the presence of the finger domain and kringle 2 that are involved with fibrin interaction (Rijken 1988). Similar to the protein monomer, tenecteplase single chain protein can be determined using size exclusion chromatography. Furthermore, the same column is suitable for both assays (Semba et al. 2003; Lentz, Joyce & Lam 2011).

1.2.3.5. *In vitro* bioactivity

The importance of not only determining the change in drug concentration over time, but also efficacy, is highlighted by a study conducted by Lentz, Joyce and Lam (2011). Although no

change in tenecteplase concentration was detected for all storage vessels, there was a significant decrease in bioactivity observed; tenecteplase was found to be compatible with glass vials and polyurethane and silicone central venous catheters for up to 72 hours at 37°C, and polysulfone-based central venous access ports for up to 24 hours (Lentz, Joyce & Lam 2011).

A microplate-based clot lysis assay has been utilised by Lentz, Joyce & Lam (2011) and Semba et al. (2003) to determine the *in vitro* bioactivity of tenecteplase. This assay evaluates the time taken for a fixed concentration of tenecteplase to dissolve a clot containing fibrinogen and thrombin in the presence of plasminogen, with the clot lysis time directly relating to the specified tenecteplase concentration (Lentz, Joyce & Lam 2011). The method involves diluting the tenecteplase samples in buffer and assessing the turbidity using a microplate spectrophotometer (Lentz, Joyce & Lam 2011).

A simpler method for studying clot lysis of thrombolytic drugs *in vitro* was developed by Prasad et al. (2006). Whole blood was allowed to form clots in pre-weighed sterile microcentrifuge tubes, the serum removed, and the clot weighed. Following lysis with four concentrations of the thrombolytic drug streptokinase, fluid was removed and the clot weighed again, with percentage of clot lysis calculated using the difference in tube weight before and after clot lysis (Prasad et al. 2006). It was found that the clot lytic activity of streptokinase (ranging from a clot lysis percentage of 62 – 71%) was significantly different to that of water (clot weight difference of 2.55%) (Prasad et al. 2006). This method could easily be applied to testing the clot lytic activity of other thrombolytic drugs such as tenecteplase.

1.2.3.6. Fibrin degradation product

When observing the lytic activity of plasminogen activators, it is important to not rely exclusively on clot weight. A study by Elnager et al. (2014) found that, while increasing the concentration of streptokinase applied to whole blood clots did not have much of an influence on clot weight, D-dimer concentration was significantly affected. The analysis of D-dimer, the primary product produced during the degradation of thrombin-generated fibrin clots, is useful for determining the efficacy of tenecteplase. The presence of D-dimer indicates blood-clotting activity and fibrin degradation has been initiated as it is one of the final products of complete fibrinolysis (Ridker et al. 1994; Brügger-Andersen et al. 2007). Lew et al. (1986) found that there was no detectable serum level of D-dimer in patients with AMI on admission to hospital, however these levels were elevated after administration of an intravenous thrombolytic drug

and persisted for more than 12 hours in most patients. Wang et al. (2002) also found D-dimer levels increased markedly following thrombolytic therapy. Specifically, Melzer et al. (2004) showed tenecteplase administered to patients with massive and submassive PE resulted in markedly increased D-dimer concentration within 20 – 120 minutes, while Brügger-Andersen et al. (2007) saw increased D-dimer in blood removed from patients with ST-elevation myocardial infarction (STEMI) following tenecteplase administration. These studies indicate that assessing serum D-dimer is suitable for determining tenecteplase efficacy, and that an increase in D-dimer would suggest some level of thrombolysis has occurred.

Recent adaptations to central laboratory-based quantitative D-dimer assays have seen them move from enzyme-linked immunosorbent assays (ELISAs) to highly sensitive coagulation and clinical chemistry analysers. Automated microparticle assays performed on a coagulation analyser, such as latex- and particle-enhanced immunoturbidimetry, are both rapid and as analytically sensitive as conventional ELISA (Riley et al. 2016). A beam of light is passed through a suspension of latex or polystyrene microparticles coated with monoclonal antibodies (mAb) specific for D-dimer epitopes. There are a variety of mAb available (more than twenty are used in over thirty D-dimer assays), and they differ in their epitopal specificity (Reber & De Moerloose 2000; Longstaff et al. 2016). When plasma is added to the suspension, any D-dimer present is captured by the mAb and immobilised, resulting in agglutination of the microparticles (Linkins & Takach Lapner, 2017). This effect increases absorbance of light that is measured photometrically, and correlates directly to the amount of D-dimer present (Riley et al. 2016; Innovance D-Dimer Package Insert 2017). Numerous studies have used the immunoturbidimetric assay to determine clot lytic activity of thrombolytics, including tenecteplase specifically (Lew et al. 1986; Brügger-Andersen et al. 2007; Elnager et al. 2014).

1.3. STUDY C – The stability of EBC-46

1.3.1. EBC-46

EBC-46 (Tigilanol tiglate, 12-Tigloyl-13-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one; $C_{30}H_{42}O_{10}$; 562.65 g/mol) is a novel diterpene ester and protein kinase C (PKC) activating compound, particularly the –β isoforms. It is currently being developed for the intralesional treatment of solid tumours, the topical treatment of complicated acute or chronic non-healing wounds, and potentially as a chemotherapeutic topical agent for

the treatment of non-melanoma skin cancers (Boyle et al. 2014; Nufer, Yamada & Prow 2016; Dally et al. 2017). Isolated from the kernels of the fruit of Blushwood (*Fontainea picrosperma*), a small dioecious tree indigenous to North Queensland's Atherton Tablelands, it is under development by QBiotics Pty. Ltd. (Adams 2013; Boyle et al. 2014; Grant et al. 2017). Although similar in structure to the prototypic PKC-activating compound phorbol 12-myristate 13-acetate (PMA) (Figure 1.6), EBC-46 is less hydrophobic due to short ester side-chains and hydroxylation of the B ring (Boyle et al. 2014). Direct injection allows for higher local drug concentrations and decreased toxicity compared to systemic agents, while topical application offers a non-invasive and less-destructive alternative to traditional skin cancer therapy (Boyle et al. 2014; Nufer, Yamada & Prow 2016). Recent trials in previously untreatable dogs, cats and horses have also shown promising results for EBC-46 in the local treatment of a range of tumour types, including mast cell tumours, melanomas, squamous cell carcinomas, adenocarcinomas, soft tissue sarcomas, myxosarcomas, nasosinal facial ulcerations and equine sarcoids. Destruction of the tumour has been found to occur within days, with rapid healing of the site and no significant adverse effects when used at therapeutic doses (Campbell et al. 2014).

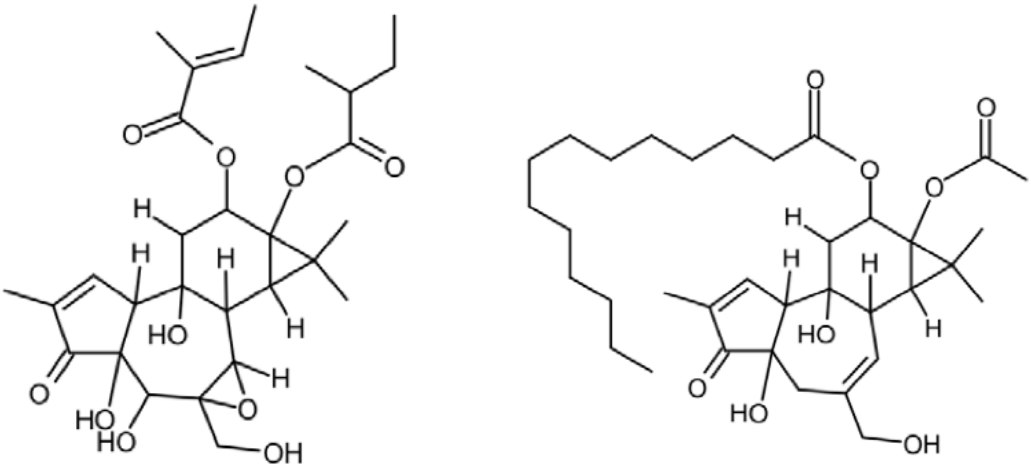

Figure 1.6. Structure of EBC-46 (left) and PMA (right).
Source: Boyle et al. 2014

PKC isoenzymes are known to be involved in and regulate many cellular functions, including gene transcription/translation, cell shape, cell death, cellular proliferation, and vasculature formation, the latter two being important for tumour growth and metastasis (Mochly-Rosen, Das & Grimes 2012). As PKC isoenzymes are overexpressed during the course of a range of cancer types, inhibition of PKC signalling has been identified as a viable anti-cancer treatment

(Totoń et al. 2011). However, pre-clinical research has uncovered challenges with potential PKC-inhibiting targets for drug development, and therefore disappointing outcomes (Mochly-Rosen, Das & Grimes 2012). For example, enzastaurin, an orally available selective inhibitor of PKC-β, showed inadequate efficacy during Phase II studies in patients with a number of cancer types including lymphomas and non-small cell lung cancer (Mochly-Rosen, Das & Grimes 2012). Conversely, research into the effect of intravenously administering PKC-activating compounds has shown promising results, including the induction of endothelial cell activation, increased permeability of endothelial cells, and the swelling and disruption of tumour vessel morphology that suggests specificity for tumour cells (Nufer, Yamada & Prow 2016). Treatment with PMA produced remission of chemotherapy-resistant myleocytic malignancies, and ingenol 3-angelate (PEP005) achieved enduring regression of skin cancer lesions, including melanoma (Han et al. 1998; Ogbourne et al. 2004). It also recently showed efficacy in squamous cell carcinoma models (Cozzi et al. 2013).

A recent study by Boyle et al. (2014) determined the effectiveness of intra-lesional injection of EBC-46 in pre-clinical models of cancer, both in human cells and in mice. When compared to PMA, it was found that EBC-46 differed in its selectivity and/or potency towards specific isoforms. Additionally, where all treated sites relapsed following PMA injection, EBC-46 was threefold less potent for cell killing *in vitro* but was more effective *in vivo*, with a cure rate of more than 70% (Boyle et al. 2014). Following a single intra-tumoural or sub-cutaneous injection into tumour-bearing and non-tumour bearing mice, EBC-46 was found to be retained at the tumour site as a result of tumour wall swelling, while also showing specificity of vascular damage within tumour sites compared to normal skin, leading to anti-cancer efficacy (Boyle et al. 2014). This effect is beneficial, as targeted drug accumulation allows controlled delivery to specific lesions while preventing unwanted effects in healthy tissues (Nufer, Yamada & Prow 2016). Specifically, the study found EBC-46 caused PKC-dependent haemorrhagic necrosis in subcutaneous tumours within 24 hours, with immunostaining for the endothelial marker CD31 showing substantial vascular disruption of tumour tissue (Lickliter et al. 2015). There were no viable tumour cells found 4 hours after EBC-46 injection by *ex vivo* culture, with the tumour nuclei becoming pale and shrunken after 2 – 4 hours and disorganisation of the tumour cells occurring after 4 – 8 hours (Boyle et al. 2014). In addition to direct disruption of tumour vasculature, rapid necrosis by EBC-46 was also found to be the result of acute but highly localised pro-inflammatory responses, and local recruitment and activation of leukocytes (Campbell et al. 2014).

When trialled in domesticated animals with tumours unsuitable for current standard of care, the anticancer properties of EBC-46 were also accompanied by the stimulation of exceptional dermal wound healing, including enhanced wound reepithelialisation, accelerated closure, and reduced scarring (Campbell et al. 2014). Specifically, a 2017 study by Campbell, Poulos and Lowden investigated the use of a combination of the corticosteroid triamcinolone and EBC-46 in treating a dog with a large subcutaneous mast cell tumour, a common neoplastic disease in canines generally controlled by surgery. It was found that following intra-tumoural injection of EBC-46, the tumour was not only effectively destroyed within 7 days of treatment without the need for sedation or anaesthesia, but the wound formed as a result of tumour destruction was almost completely healed 42 days post-treatment (Campbell, Poulos & Lowden 2017). There was also a case reported by Hansen et al. (2017) of a dog diagnosed with a severe case of canine papillomavirus infection who was treated with two applications of a topical gel formation of tigilanol tiglate. Four weeks following the second treatment, there was almost complete resolution of the lesions on the treated area, with no detrimental effect to unaffected skin between lesions and only limited local discomfort (Hansen et al. 2017). Hansen et al. (2017) also reported similar efficacy in other unpublished cases of feline viral plaques and Bowenoid squamous cell carcinoma *in* situ, and equine sarcoids. These exceptional wound-healing properties are believed to be a result of stimulated keratinocyte proliferation, by both EBC-46 and its less-active analogue EBC-211 (12-tigloyl-13-(2-methylbutanoyl)-5,6-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one) (Moses et al. 2014). All of these results suggest the potential for EBC-46 as not only an anticancer agent, but also as a novel wound healing pharmaceutical (Campbell et al. 2014).

Further development of EBC-46 in the management of canine solid tumours is now underway following a recent study that determined an efficacious, well-tolerated dose with only mild and transient adverse events. As a result, present studies are exploring the efficacy of EBC-46 in treating canine mast cell tumours and soft tissue sarcomas (Miller et al. 2019). Additionally, a Human Clinical Phase IIA efficacy trial is currently planned following positive outcomes in recently completed Phase I/IIA human safety and tolerability clinical trials (QBiotics Group Limited 2018). The growing interest in the biostability of EBC-46 and its derivatives makes ensuring a stable, purified sample especially important. Previous investigations by researchers at QIMR Berghofer Medical Research Institute (through support and funding from QBiotics) found that the formation of two less-active EBC-46 degradation products appears to be influenced by pH: at low pH (<5) EBC-158 is formed through hydrolysis of the 6,7-epoxide

in the B ring of the parent compound, while at higher pH (>5) EBC-211 is produced via migration of the epoxide (a type of Payne rearrangement) to the 5,6-position (Figure 1.7).

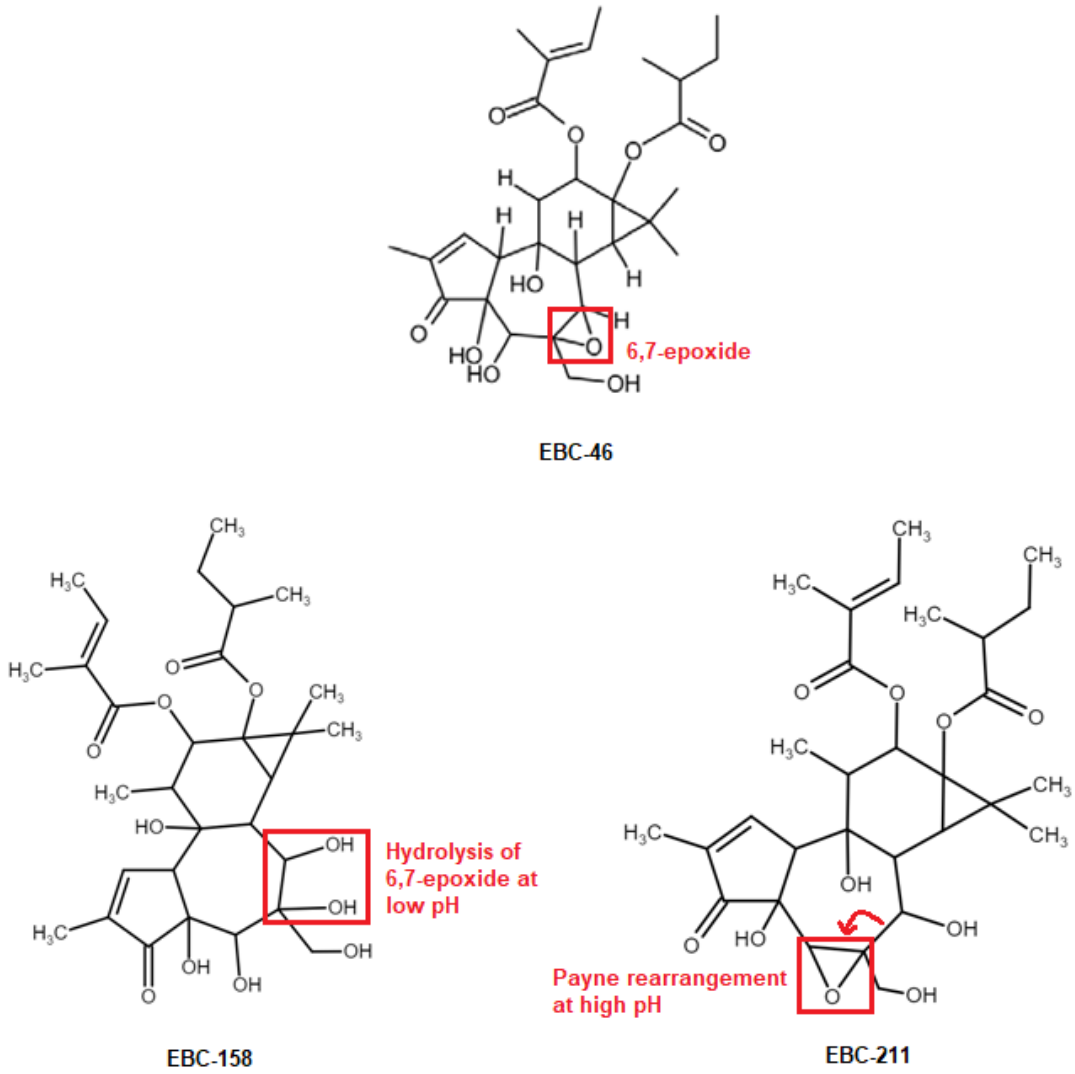

Figure 1.7. The formation of EBC-46 degradation products, EBC-158 and EBC-211, under different pH conditions.

Exposing drugs to different environmental and chemical conditions, and determining how they degrade (e.g. identifying metabolites), are essential components of the development phase; they indicate appropriate storage conditions and formulation requirements to ensure maximum stability and efficacy. As EBC-46 is a novel compound that is still early in its development, these stability studies have not been extensively performed. It is imperative these studies are performed early in a drug's development as they can advise possible

excipients that should be included or excluded from the final formulation, and how the drug should be stored to ensure it remains effective for subsequent efficacy research. Therefore, the aim of this study was to determine the effect of temperature, light, air and pH on the stability of EBC-46 in storage over time, as well as looking at the metabolism of EBC-46 in an *ex vivo* model, in order to provide valuable insight into how best to store the purified product to ensure its long-term stability.

1.3.2. EBC-46 stability and analysis techniques

As stated previously, EBC-46 is a novel drug still under development and stability studies have not yet been performed. Therefore, specific details on analysis techniques are not readily available. However, this investigation was conducted alongside researchers at QIMR Berghofer Medical Research Institute in Brisbane, who have been involved in numerous preliminary studies of EBC-46 efficacy. As a consequence, they have developed their own HPLC methods for EBC-46 analysis and these were utilised for this study. To provide a comprehensive overview of stability, EBC-46 was subjected to factors commonly known to affect drugs and their metabolites, namely temperature, light, oxidation, pH, and enzymatic degradation (Briscoe & Hage 2009).

1.4. Investigation aims

The aims of this investigation, comprising studies A, B and C, is to ascertain how different storage conditions impact upon the stability of remifentanil, propofol, tenecteplase, and EBC-46. Manufacturers provide basic storage instructions for their compounds, such as storage temperature and light exposure, but the consequences of even minor fluctuations from these recommendations is not always clear. If not properly monitored or controlled, conditions encountered during drug transport, storage, and use could induce chemical processes (including oxidation and hydrolysis) that alter the conformational structure, potentially compromising pharmacological efficacy and patient safety.

Each study assessed the stability of different drugs under a range of storage conditions. Specifically, the key aims of each study are as follows:

- **Study A – The stability of remifentanil and propofol in combination**:
  Determine how modifying concentration, pH, and electrolyte concentration (through choice of diluent) of a remifentanil and propofol mixture affects their stability, and investigate if a compatible combination of the drugs is possible that would allow for efficient simultaneous infusion.
- **Study B – The stability of lyophilised tenecteplase at high temperatures:**
  Examine how temperatures exceeding manufacturer's suggestions that are encountered during ambulance transport and storage affect the stability and pharmacological efficacy of tenecteplase, and determine if measures should be implemented to ensure patient wellbeing is not compromised.
- **Study C – The stability of EBC-46:**
  Explore the effect of altering temperature, pH, light exposure, and oxygen presence on the stability and efficacy of EBC-46 to uncover the most appropriate conditions for preserving potency of the pure product during storage, as well as elucidate potential metabolites that may occur *in vivo*.

These studies provide valuable information to both clinicians and researchers on the importance of controlling the storage conditions of pharmaceuticals. It is anticipated that the findings will inform best practice for those involved with the specific drugs investigated, and elicit improvements in their storage and administration. However, it is hoped that the research will be more far-reaching, and is applied to other similar pharmaceuticals to ensure drug potency and patient wellbeing are preserved.

2. STUDY A – THE STABILITY OF REMIFENTANIL AND PROPOFOL IN COMBINATION

2.1. Chapter summary

This study examines the stability of remifentanil and propofol when used in combination for simultaneous infusion, and determines the effect of manipulating concentration, pH, and electrolyte concentration on their compatibility when stored as a mixture. In doing so, it elucidates the significance of the storage environment to the stability of remifentanil and propofol, and indicates how their stability as a mixture might be improved through small modifications to specific storage conditions. This has the potential to establish simultaneous infusion as an efficient, effective, and safe way of administering remifentanil and propofol to patients, thereby challenging the longstanding perception that these drugs are incompatible.

Propofol is a sedative intravenous (IV) agent used for the induction and maintenance of general anaesthesia in both surgical and intensive care settings. Remifentanil is an analgesic that is most commonly administered intravenously (following reconstitution from its powdered form) in conjunction with an anaesthetic agent. Both drugs have rapid onset of action and elimination, and when used in combination they exhibit a synergism that allows more manageable control over patient comfort. Typically, remifentanil and propofol are administered through separate IV lines; it is advised by remifentanil manufacturers that it not be mixed with propofol in the same IV line. However, no further explanation is provided as to why this is the case. Clinicians from the Rockhampton Hospital approached the research team as they were interested in conducting a study to determine if remifentanil and propofol could be mixed prior to patient administration. Admixing the drugs provides benefits such as ease-of-use, cost effectiveness, and increased availability of IV lines for other drugs the patient may require. However, it is critical to first establish the stability of drugs when mixed and stored, as incompatibility could potentially decrease the concentration of active ingredients, modify or degrade drug structure, affect solution homogeneity through the formation of precipitates (potentially blocking IV lines), and ultimately put the patient at risk of harm.

To ascertain the compatibility of remifentanil and propofol in this study, the effect of concentration, solution pH, and diluent used for remifentanil resuspension on the degradation of each drug was explored over a period of 24 hours. While drugs would not be used for this length of time clinically, this element was included in the investigation to support fundamental chemical principles of degradation and allow a more complete profile of stability to be

obtained. This was also achieved by examining both drugs in isolation and when mixed. Concentration was deemed a significant factor for this combination as the amount of remifentanil required is much less than that of propofol. Solution pH and the diluent used are known to potentially alter degradation reactions, and as remifentanil hydrolyses under certain conditions it was important to consider how the presence of propofol would impact this. The technique of high-performance liquid chromatography was used to analyse drug concentration throughout this study. Further information on the methods utilised for this study can be found in the Appendix of this thesis (see section 7.1.2).

Determining the effect of concentration, pH, and choice of diluent on the stability of remifentanil and propofol provides valuable information to clinicians on the suitability of mixing these drugs for simultaneous infusion. The findings of this study may not only introduce a more efficient method of administering remifentanil and propofol to patients, but could also initiate further investigation into how the preparation and delivery of similar medications might be improved.

The following manuscript prepared from this investigation has been published in *BMC Anesthesiology* (the citation can be found in the List of Relevant Publications section of this thesis).

The effect of concentration, reconstitution solution and pH on the stability of a remifentanil hydrochloride and propofol admixture for simultaneous co-infusion

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Abstract

Background: There are scenarios where pre-mixing and infusing analgesic and anaesthetic agents as a single intravenous (IV) solution is highly desirable; however, it is important to ensure the agents are compatible when mixed. As such, the long-term stability of a remifentanil-propofol mixture, and means of improving this, were assessed across a range of remifentanil concentrations, diluents, and time points. **Methods:** Remifentanil was reconstituted with ultrapure water, 0.9% saline, 20% saline, or 8.4% sodium bicarbonate solution (the latter two chosen for their pH characteristics, rather than their use in pharmaceutical reconstitution) and then mixed with propofol (1%) or further diluted with water to derive concentrations of 10 – 50 μg mL^{-1}. Remifentanil and propofol concentrations were determined initially and then periodically for up to 24 hours using high performance liquid chromatography (HPLC). Mass spectrometry (MS) was used to detect degradation products in solutions containing 30 μg mL^{-1} of remifentanil. Statistical analysis was performed using ANOVA and Student's *t*-test, with a significance value of 0.05. **Results:** Isolated remifentanil (pH <4) and propofol (pH 7.35) did not degrade significantly when reconstituted with water or saline solution over 24 hours, while remifentanil reconstituted with sodium bicarbonate degraded significantly ($P<0.001$, pH 8.65). Mixing with propofol substantially increased the pH of the mixture and resulted in significant remifentanil degradation for all reconstitution solutions used, while propofol remained stable (pH 6.50). The amount of degradation product detected in samples containing isolated remifentanil and a mixture of the drugs was proportional to the remifentanil degradation observed. **Conclusions:** Remifentanil stability is affected by both the reconstitution solution used and when mixed with propofol, with pH appearing to be a contributing factor to degradation. If the pH of the solution and concentration of remifentanil are correctly controlled, e.g. through the use of a more acidic diluent, an admixture of remifentanil and propofol may be useful clinically.

Keywords: Chemical Stability; Drug-drug interaction(s); HPLC; Pharmaceutical Preparations; Propofol; Remifentanil.

Background

Optimisation of both analgesia and sedation is vital to ensure adequate pain control, minimise agitation and anxiety, facilitate patient compliance for mechanical ventilation or diagnostic interventions, and provide patient comfort [1]. Most patients receiving invasive ventilation are on several continuous infusions, often achieved using separate or multi-channel infusion pumps, IV connectors, and/or multi-lumen catheters [2]. Even in well-equipped hospital settings (e.g. intensive care units) this can be a complex undertaking, with numerous potential correlated patient safety risks such as separate drugs running at incorrect infusion rates and required medications being connected to the infusion system but not administered to the patient [3]. However, there are scenarios outside of a sophisticated medical facility environment where the availability of specialised equipment may be limited, or many patients must be attended to within a short schedule. This can include those in rural or remote locations, a busy ambulatory surgery centre or office practice, or military medical personnel attending to wounded soldiers in harsh environments [4,5]. In all instances, it may be advantageous to pre-mix infusion agents and administer them via a simplified, single IV infusion. The kinetics of such infusions is not always well-understood.

Remifentanil is a highly potent analgesic agent. It is an ultra-short-acting mu-opioid receptor agonist that undergoes organ independent metabolism by blood and non-specific tissue esterases, forming an inactive metabolite [6,7]. It has a rapid onset of effect, with maximum ventilatory depression occurring approximately 2 - 3 minutes following administration of an initial bolus [8]. The context sensitive half-life (the time taken for blood concentration to decrease by 50% following termination of a continuous infusion that maintained a steady-state concentration) is around 3 minutes even after prolonged infusions, with no significant drug accumulation [9,10]. This is a point of difference from other commonly used analgesic agents, where the duration of infusion or renal impairment may impact on duration of effect. This pharmacokinetic profile may be clinically advantageous in a variety of patients.

Propofol is a commonly-used short-acting sedative agent with rapid onset of anaesthesia (only a few seconds), duration of effect (3–5 minutes), and recovery [11]. The mechanism of action is via positive modulation of the inhibitory function of the gamma-aminobutyric acid (GABA) neurotransmitter through $GABA_A$ receptors [12]. Using a combination of remifentanil and propofol can offer several advantages in a clinical setting [13,14]. The short duration of pharmacological action shared by remifentanil and propofol may allow for improved control

over pain and anaesthesia management and afford faster recovery for patients, while the synergistic relationship exhibited when the drugs are co-administered reduces remifentanil and propofol requirements [4,14].

Manufacturers of remifentanil advise against mixing with propofol; however, further explanation is not provided. The utility of a remifentanil-propofol admixture has already been explored in areas such as radiation therapy, dental extraction, and paediatric and elective outpatient surgeries, while a current clinical trial is investigating the use of a remifentanil-propofol mixture for breast cancer surgery [4,15–19]. Previous studies have concluded that while simultaneous infusion removed the ability to selectively control the use of each drug, it resulted in decreased incidences of procedural respiratory depression and patient recovery time [18,19]. Furthermore, when mixed with propofol, remifentanil has been found to inhibit the bacterial growth that readily occurs within the lipid emulsion, possibly due to its glycine excipient and low pH [20,21].

When admixing remifentanil and propofol for simultaneous infusion, it is crucial to understand their compatibility from a chemical perspective, including the effect of any interactions on stability and efficacy. If degradation unknowingly occurs, an unpredictable response may arise and patient care may be compromised. Research in this area could potentially lead to methods of improving the stability of the mixture and ensure its safety and effectiveness, in addition to providing avenues for the use of other combinations of opiate agonists and short-acting anaesthetics. As propofol is an opaque liquid, it is difficult to detect incompatibility or any changes in solution stability through visual assessment alone. In addition, the organ-independent metabolism of remifentanil combined with its short duration of effect mandate careful evaluation of stability of the parent compound in any mixture or co-infusion.

Previous studies have, to different degrees, considered factors such as the storage vessel and drug concentration when exploring the stability of a remifentanil and propofol mixture; most investigations include admixtures containing two or three concentrations of remifentanil and/or propofol that are stored in polyvinyl chloride and propylene vessels [22,23]. For mixtures of remifentanil and propofol specifically, there are a lack of studies investigating a variety of remifentanil concentrations, the impact of mixing on both remifentanil and propofol concentration, and how manipulating solution pH (through the use of different remifentanil reconstitution mediums) affects the stability of the mixture. pH is of particular importance for a combination of remifentanil and propofol as remifentanil is believed to undergo rapid

hydrolysis when exposed to a pH range of 7 – 7.5 [21]. After reconstitution with water, remifentanil has a pH of 3.0 [24]. In comparison, propofol can have a pH ranging from 6 to 8.5 [25].

The aim of this study was to determine the stability of both remifentanil and propofol solutions, alone and in combination, when stored in glass over 24 hours, and ascertain if drug concentration, diluent used, or pH could be altered to improve their stability from a pharmaceutic perspective. This could indicate if remifentanil and propofol are compatible to be pre-mixed and infused as a single intravenous solution. The drugs are considered stable in this study as long as the concentration exceeds the minimum acceptable potency level of 90% [46].

Methods

Materials and reagents

Remifentanil hydrochloride ("Ultiva for Injection" from GlaxoSmithKline Australia Pty Ltd, Boronia, VIC, Australia, and "DBL Remifentanil powder for injection" from Hospira Pty Ltd, Melbourne, VIC, Australia), Propofol (containing propofol (10 mg mL^{-1}), soya oil (100 mg mL^{-1}), glycerol (22.5 mg mL^{-1}), egg lecithin (12 mg mL^{-1}), sodium oleate (0.3 mg mL^{-1}); "Propofol Sandoz" from Sandoz Pty Ltd, Pyrmont, NSW, Australia, and "Provive 1%" from Claris Lifesciences (Aust) Pty Limited, Burwood, NSW, Australia), 0.9% saline solution, 20% saline solution and 8.4% sodium bicarbonate solution were all of clinical grade and donated by the Rockhampton Hospital Pharmacy Department (Rockhampton, QLD, Australia). Methanol, acetonitrile and ammonium acetate were purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia). All chemicals were ACS analytical grade or HPLC grade. Ultrapure water was prepared by a Milli-Q® Reference Water Purification System (Merck Millipore, Bayswater, VIC, Australia).

Instrumentation

All samples were analysed using an Agilent Technologies 1200 series HPLC (Agilent Technologies, Melbourne, VIC, Australia) equipped with a variable wavelength diode array detector set at 210 nm and 270 nm for remifentanil and propofol analysis, respectively. The remifentanil protocol used an Agilent Eclipse XDB-C18 column with dimensions of 150 x 4.6 mm with a particle size of 5 µm, and a mobile phase of 75% methanol and 25% 10 mM ammonium acetate (flow rate 1.5 mL min^{-1}) [26-28]. The protocol for propofol analysis used an Agilent Eclipse XDB-C18 column with dimensions of 250 x 4.6 mm and a particle size of 5 µm. The mobile phase consisted of 65% acetonitrile and 35% water with a flow rate of 2.0 mL min^{-1} [29,30]. Solution pH was determined using a Eutech Instruments 700 pH meter (Eutech Instruments Pte Ltd, Singapore). Subsequent assays were performed on the same samples at 1, 2, 3, 4, 6, 12 and 24 hours following preparation.

For HPLC-MS analysis, a Prominence HPLC system (Shimadzu Scientific Instruments, Rydalmere, NSW, Australia) coupled to an API3200 LC-MS/MS mass spectrometer (Applied Biosystems/MDS Analytical Technologies/SCIEX, Mt Waverley, VIC, Australia) was utilised. Separation was achieved using an Agilent Zorbax SB C18 column with dimensions of 150 x 4.6 mm and a particle size of 5 µm, and a mobile phase of 60% methanol and 40% 10 mM

ammonium acetate (flow rate 1.3 mL min^{-1}). The MS system was run in the positive ion mode using nitrogen as the desolvation gas.

Sample preparation

Triplicate 5 mg preparations of remifentanil hydrochloride for injection were reconstituted with 5 mL of either ultrapure water, 0.9% saline solution, 20% saline solution, or 8.4% sodium bicarbonate solution (the latter two chosen for their pH characteristics, rather than their use in pharmaceutical reconstitution). Samples were then added to 10 mg mL^{-1} propofol for injection (final propofol concentration of 9.5 mg mL^{-1}) or left in isolation by mixing with ultrapure water to produce a solution with a final remifentanil concentration of 50 µg mL^{-1}. This procedure was repeated to give solutions with final remifentanil concentrations of 40, 30, 20 and 10 µg mL^{-1} (final propofol concentrations of 9.6, 9.7, 9.8 and 9.9 mg mL^{-1}).

To determine propofol degradation in isolation, triplicate volumes of 1 – 5 mL of ultrapure water, 0.9% saline solution and 20% saline solution were added to 10 mg mL^{-1} propofol for final propofol concentrations of 9.5 – 9.9 mg mL^{-1}.

Immediately following preparation and pH determination, the remifentanil and propofol concentration in each sample was assessed in emulsion using HPLC. Samples demonstrating significant remifentanil deterioration were analysed further for the presence of degradation products using HPLC-MS. Subsequent assays were taken 1, 2, 3, 4, 6, 12 and 24 hours following preparation. All samples were stored at room temperature (22°C – 24°C) between assays, and inverted prior to aliquot removal to prevent mixture separation and reduce the influence of oil droplet flocculation/creaming [31,32].

Statistical analysis

Statistical analysis was performed using ANOVA and Student's *t*-test where appropriate, with results deemed significant when $P \leq 0.05$ (Prism version 4.02; GraphPad Software, San Diego, CA, USA).

Results

Remifentanil in isolation

No obvious precipitate was formed in any of the remifentanil solutions over time. Remifentanil in isolation did not degrade significantly over 24 hours when reconstituted with either water, 0.9% saline solution or 20% saline solution; all concentrations contained more than 92% of the original remifentanil after 24 hours, exceeding the minimum acceptable potency level of 90% (Figure 1). The pH of these solutions over 24 hours were also similar, averaging 3.74, 3.94, and 3.95 for remifentanil reconstituted with water, 0.9% saline and 20% saline, respectively (see Additional file 1).

Remifentanil reconstituted with sodium bicarbonate solution degraded rapidly, with no remifentanil remaining after 24 hours (Figure 1). Furthermore, compared to the other reconstitution solutions, sodium bicarbonate had significant effects on remifentanil degradation ($P<0.01$) after only 1 hour for all concentrations. The pH of the sodium bicarbonate solutions averaged 8.65 over time (see Additional file 1).

Propofol in isolation

Propofol did not degrade significantly over 24 hours when in isolation or after the addition of water, 0.9% saline solution, or 20% saline solution, with all solutions having more than 97% of the original propofol remaining after 24 hours (Table 1). There were no obvious visual signs of propofol emulsion instability or separation following the addition of the diluents over the time period tested. The pH of propofol in isolation and after mixing with water did not change over 24 hours (pH = 7.70). These solutions had a significantly greater pH than propofol mixed with 0.9% saline solution (average pH of 7.40, $P<0.0001$) and 20% saline solution (average pH of 6.98, $P<0.0001$) (Table 1).

(Insert Table 1 here)

Remifentanil-propofol mixture

There were no obvious visual incompatibilities or signs of emulsion instability/separation when remifentanil and propofol were mixed over the time period tested.

Remifentanil showed significant degradation when mixed with propofol. The percentage of remifentanil remaining after reconstituting with water or 0.9% saline and then mixing with

propofol decreased by 50 – 60% over 24 hours, compared to the same remifentanil solutions in isolation. These solutions also had the highest pH readings over 24 hours, with averages of 6.86 for 0.9% saline-reconstituted solutions and 6.96 for water-reconstituted solutions (Table 2). Propofol in these solutions, as well as those containing remifentanil reconstituted with 20% saline solution, remained stable, with all solutions having greater than 96% of the original propofol remaining after 24 hours (Table 2).

Concentration did not impact on the stability of water-reconstituted remifentanil mixed with propofol. Solutions containing 30 μg mL^{-1} of remifentanil had significantly more propofol than all others after 24 hours ($P<0.05$), but this difference was only 1.2% greater than the next highest concentration (Table 2). For remifentanil reconstituted with 0.9% saline and mixed with propofol, a concentration of 50 μg mL^{-1} was significantly more stable ($P<0.01$) than every other concentration after 24 hours (Table 2), with significant differences ($P<0.03$) apparent between 50 μg mL^{-1} and 10, 20 and 40 μg mL^{-1} concentrations after 6 hours. Solutions containing 50 μg mL^{-1} remifentanil also had the lowest average pH over 24 hours of 6.50. Remifentanil concentration had no statistically significant effect on propofol degradation in 0.9% saline mixtures (Table 2).

Remifentanil reconstituted with 20% saline and mixed with propofol showed the least degradation compared to the same remifentanil solutions in isolation, with 46 – 60% of the original remaining after 24 hours. These solutions also had significantly lower ($P<0.0001$) pH readings of all reconstitution solutions tested, with an overall average of 6.60 over 24 hours (Table 2). Similarly, the 20% saline mixtures with the most stable remifentanil concentrations also had the lowest average pH over 24 hours; solutions containing 40 and 50 μg mL^{-1} of remifentanil were significantly more stable ($P<0.03$) than those with 10, 20 and 30 μg mL^{-1} from 12 hours onwards (Table 2). However, propofol in mixtures with 30, 40 and 50 μg mL^{-1} of remifentanil reconstituted with 20% saline solution were significantly less stable than those containing 10 μg mL^{-1} of remifentanil after 24 hours ($P<0.05$) (Table 2).

(Insert Table 2 here)

Remifentanil degradation product

A degradation product with an ion weight of 362 Da was formed over 24 hours in all samples analysed containing remifentanil. Solutions containing 30 μg mL^{-1} of remifentanil reconstituted with sodium bicarbonate solution in isolation produced the highest concentration of the

degradation product ($P<0.0001$), with 33.7 μg mL^{-1} detectable after 24 hours (Figure 2). Interestingly, this combination also resulted in the greatest remifentanil degradation of the samples analysed. Samples containing 30 μg mL^{-1} of remifentanil in propofol that were reconstituted with water and 0.9% saline exhibited a similar increase in the degradation product, with 22.1 μg mL^{-1} and 22.0 μg mL^{-1} detected after 24 hours, respectively. Both solutions contained significantly more degradation product ($P<0.021$) than 20% saline-reconstituted solutions, with 19.9 μg mL^{-1} detected after 24 hours (Figure 2).

Discussion

This study demonstrated that the influence of reconstitution medium, pH and drug concentration is important for the stability of a remifentanil-propofol solution. The stability of remifentanil following reconstitution is more affected by the pH of the reconstitution medium than the initial remifentanil concentration. Remifentanil degraded significantly when mixed with propofol. Concentration had more of an effect on remifentanil degradation in the mixture, with greater stability observed at higher remifentanil concentrations. The initial concentration of remifentanil was also found to impact on propofol degradation, with less stability observed as the amount of remifentanil increased. In all cases, however, the degradation seen appears to be affected by the influence of initial remifentanil concentration on solution pH, as solutions containing 50 μg mL^{-1} of remifentanil had a lower overall pH than those containing 10 μg mL^{-1} of remifentanil. Interestingly, these findings correspond with the suggested infusion concentration of 50 μg mL^{-1} provided by the manufacturers of remifentanil hydrochloride. For all remifentanil solutions (isolated and mixed), elevated pH resulted in increased formation of degradation products.

A comparable study from Stewart et al. investigated the stability of high (50 μg mL^{-1}) and low (5 μg mL^{-1}) concentrations of remifentanil in 10 mg mL^{-1} of propofol when stored in polyvinyl chloride bags and propylene syringes [22]. Similar to our results, they demonstrated that both drugs in isolation remained stable while the mixture did not, the higher remifentanil concentration had greater stability than the low concentration, propofol was more stable in isolation than when mixed with remifentanil, and the storage conditions have a greater influence on propofol stability than the initial remifentanil concentration added to the mixture [22]. Another similar study by Gersonde, Eisend, Haake and Kunze investigated the physicochemical compatibility and emulsion stability of propofol when mixed and stored with other sedatives and analgesics, including remifentanil, in a syringe for a period of 7 days [23]. All solutions were reconstituted and diluted with 0.9% NaCl, and mixed at ratios of 10:1 (v/v), 1:1 (v/v) and 1:10 (v/v) using a remifentanil concentration of 0.05 mg mL^{-1} and a propofol concentration of 20 mg mL^{-1}. Comparable to our investigation, the study demonstrated that the concentration remained above 90% for the isolated drugs after 24 hours, while the mixture containing the lowest remifentanil concentration showed the greatest change in drug concentration, decreasing to below 90% within 4 hours [23]. These findings indicate that good control of pH of the remifentanil reconstitution mixture and the use of higher concentrations of remifentanil show viability as an anaesthetic dosing regimen.

While a recent study by Bedocs, Evers and Buckenmaier III concurred with our findings that propofol alone remains stable over 24 hours, in contrast, they found a mixture of propofol, ketamine and remifentanil stored in polypropylene tubes also showed no signs of degradation [5]. Ketamine is prepared in a slightly acidic solution of pH 3.5 – 5.5 [33], and its concentration in the mixtures was 200-times that of remifentanil; unfortunately, the pH of the mixtures was not determined in the study by Bedocs, Evers and Buckenmaier III, and a reduction in solution pH may have contributed to the stability seen with remifentanil in the mixtures. Our study differs from those mentioned in that we stored the solutions in glass and investigated a greater variety of reconstitution solutions and remifentanil concentrations, examining the effect on both remifentanil and propofol stability when stored in isolation and when mixed.

The effect of altering the pH of reconstituted remifentanil was investigated via the use of different reconstitution mediums that were chosen due to their pH characteristics, rather than their physiological properties or use in pharmaceutical reconstitution. However, manufacturers of remifentanil recommend both sterile water for injection and 0.9% sodium chloride injection for reconstitution.

Remifentanil reconstituted with 8.4% sodium bicarbonate solution in isolation had an average pH of 8.7 over 24 hours for all concentrations examined, and resulted in the concentration of remifentanil decreasing rapidly. This was expected, due to the rapid aqueous hydrolysis of the sterically unhindered alkyl ester that occurs at high pH [34]. Conversely, remifentanil in isolation that was reconstituted with water, 0.9% saline solution, and 20% saline solution all had an average pH below 4 over 24 hours for all concentrations tested. The remifentanil in these solutions was very stable, with over 90% of the original concentration remaining after 24 hours. These results are consistent with known remifentanil pharmaceutics and confirmed the role of pH in its degradation. Furthermore, it highlights the importance of considering the pH of any pharmaceutical that is to be mixed with remifentanil. Due to the instability of the remifentanil-sodium bicarbonate solution, it was not included in the remifentanil-propofol mixture stability study.

While remifentanil degraded significantly when mixed with propofol, those solutions reconstituted with 20% saline were found to be the most stable over 24 hours for all concentrations tested, significantly so for 40 and 50 μg mL^{-1} concentrations. We believe this is due to the pH of these solutions, as they had the lowest of all reconstitution solutions tested and were the only solutions to have an average pH below 7. Furthermore, remifentanil mixed

with propofol was most stable when the solution pH was below pH 6, particularly around pH 5.7. This demonstrates that pH is an important factor in remifentanil stability not only in isolation, but also when it is mixed with propofol; however, it cannot be concluded that pH is the only factor influencing remifentanil stability in the mixture.

We examined the solutions that demonstrated the highest degradation of remifentanil. A mid-range remifentanil concentration of 30 μg mL^{-1} was chosen for further analysis as those samples showed sufficient degradation in the previous studies. Remifentanil was visible via mass spectrometry when run in the positive ion mode, with an ion weight of 376 Da, while the degradation product had an ion weight of 362 Da. It was found that the increase in concentration of the degradation product over 24 hours was directly proportional to both the alkalinity of the solution and the degradation of remifentanil. These findings correspond to those reported by Gersonde, Eisend, Haake and Kunze [23] and suggest that the detected by-product is a degraded form of remifentanil. Due to the ion weight of the unknown compound and the rapid de-esterification experienced by remifentanil, it is speculated that this degradation product is the principal metabolite, remifentanil acid (GR90291; Figure 3) [9,36,37]. Although this metabolite, eliminated by the kidneys, may accumulate in patients with severe renal impairment [9], it is much less potent (1/4600) than its parent compound [37] and does not result in clinically-significant prolonged mu-opioid effects [38]. A minor metabolite of remifentanil, the β-elimination product (GR94219), is also produced at high pH through the "retro-Michael reaction"; however, the dominant and rapid esterase metabolism results in only approximately 1% of remifentanil being eliminated in this secondary form (Figure 3) [39]. While these degradation products may not be pharmacologically relevant for most patients, their formation does render the mixture less effective in a clinical setting and highlights the importance of understanding the chemistry associated with mixing compounds.

When in isolation and when mixed with water, 0.9% saline solution or 20% saline solution, propofol concentration remained above 90% over 24 hours regardless of diluent concentration. This is potentially important in the clinical setting, as it indicates that propofol concentration remains stable over prolonged periods of infusion.

While propofol concentration remained above 90% in all solutions tested, those containing the highest concentration of saline (20%) at the highest volume (5 mL) resulted in the greatest propofol degradation, even in isolation. These findings are supported by a previous study by Wei et al., who demonstrated that propofol was stable for six hours following dilution with large

volumes of sodium chloride (ranging from 500 mL to 800 mL in a total of 1000 mL, much greater volumes than those used in our study) [40].

Nemec, Germ, Schulz-Siegmund and Ortner found that the addition of 0.9% sodium chloride to propofol 1% in the ratio of 1:1 (v/v) resulted in a minor change in the emulsion stability [41]. Emulsions containing phospholipids such as egg lecithin are charge-stabilised and have a zeta potential that enables excellent stability under normal conditions. One factor that may lower the zeta potential and impact emulsion stability is the presence of electrolytes [30]. It has been suggested that the addition of positively-charged sodium results in neutralisation of the negatively-charged surface of the propofol emulsion oil droplets, resulting in flocculation [40]. This may explain the decreased propofol stability, albeit minor, that was observed following the addition of 20% saline in our study. It should be noted that a combination of propofol and saline solution has been investigated for use in several clinical applications, including reducing pain on injection and decreasing the incidence and severity of excitatory reactions during induction of anaesthesia in young children [42,43].

While the large decrease in solution pH following the addition of remifentanil did not have a significant impact on propofol concentration specifically, pH may also affect the zeta potential, and therefore stability, of phospholipid-stabilised emulsions [44]. Therefore, the decrease in solution pH experienced following the addition of both 20% saline solution (in isolation) and remifentanil may have contributed to minor propofol emulsion destabilisation, even in mixtures where remifentanil was reconstituted with water; this may be confirmed by reconstituting remifentanil directly into the propofol emulsion. Furthermore, the decrease in pH observed over 24 hours in remifentanil-propofol mixtures may be partly attributed to the release of small amounts of free fatty acids from the propofol emulsion, as a result of phospholipid and soybean oil hydrolysis [32].

Due to the concentration degradation experienced in the remifentanil-propofol mixture, further analyses of stability, such as emulsion fat globule size/distribution, were not deemed necessary. Furthermore, these factors have been investigated in previous studies [23,41,45].

Limitations

As stated previously, the addition of saline solution may have negatively impacted upon the emulsion stability of propofol in our study. Furthermore, adding reconstituted remifentanil decreased the nominal concentration of propofol in the mixture. To determine if the decrease in propofol stability was a result of emulsion destabilisation caused by the addition of saline or reconstituted remifentanil, the mixture could be prepared by adding lyophilized remifentanil directly into the propofol emulsion (although this removes the ability to manipulate solution pH using remifentanil diluent).

As drug compatibility was a consideration when choosing diluents for this study, the pH range investigated was only quite small (with the exception of the very-alkaline sodium bicarbonate solution). This makes it difficult to get a comprehensive understanding of the most suitable pH range for maximum remifentanil-propofol mixture stability. It would be interesting to explore this further by manipulating solution pH using various buffers; however, doing so in this study would have taken the experimental parameters to a more extreme non-clinical application, potentially providing proof of concept rather than a direct clinical application. Extreme modifications of solution pH conditions would not be considered appropriate in clinical practice.

Finally, this study only considered the chemical stability of the mixture; no biological compatibility or safety tests were conducted. Therefore, any chemically-stable mixtures that show promise in subsequent stability tests should undergo emulsion globule size/distribution assays and be thoroughly scrutinised in *ex vivo* models prior to trialling in patients.

Conclusions

It is clear the stability of remifentanil is less dependent on the initial concentration and more influenced by the pH of the solution, as the addition of a neutral/alkali diluent had a negative impact on its stability. Additionally, mixing remifentanil with propofol in the same storage vessel resulted in significant remifentanil degradation. The hydrolysis of remifentanil at more alkaline pH values is likely a factor in the degradation observed in our study, and the results suggest that reconstituting remifentanil in a solution with a more acidic pH may increase its short-term storage stability. For propofol, the addition of remifentanil, water or saline solution at the concentrations tested, as well as the resulting changes in pH, did not have a significant

negative impact on its concentration when stored over 24 hours. However, our study indicates, from a chemistry perspective, that remifentanil and propofol may not be suitable to store as an admixture long-term prior to infusion.

Abbreviations

- HPLC: High Performance Liquid Chromatography
- IV: Intravenous
- MS: Mass Spectrometry
- GABA: Gamma-Aminobutyric Acid
- ANOVA: Analysis of Variance

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Author's Contributions

KB, DA, PK and AF were involved in conceptualization of the study, and along with RV, revision and editing of the paper. AF was also involved in supervision, and PK, EH, and RV

visualization, of the project, while KB was fundamental in providing resources. EH and RV were involved in project administration, methodology, and validation, while EH was also heavily involved in performing the investigation, as well as formal analysis of the data and writing the original draft of the paper. All authors have read and approve of the final version of the manuscript, and agree to be personally accountable for their own contributions and ensure that questions related to the accuracy or integrity of any part of the work, even those in which they were not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Not applicable.

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Table 1. pH and percentage of the original propofol remaining when mixed with water and saline solution

	10 μg mL^{-1}				20 μg mL^{-1}				30 μg mL^{-1}				40 μg mL^{-1}				50 μg mL^{-1}			
	1 h	**6 h**	**12 h**	**24 h**	**1 h**	**6 h**	**12 h**	**24 h**	**1 h**	**6 h**	**12 h**	**24 h**	**1 h**	**6 h**	**12 h**	**24 h**	**1 h**	**6 h**	**12 h**	**24 h**
Percent Propofol Remaining when left in Isolation (mean ± SEM; n=3)																				
I	102.3 ±0.90	101.1 ±1.03	100.5 ±1.05	100.2 ±1.04	102.3 ±0.90	101.1 ±1.03	100.5 ±1.05	100.2 ±1.04	102.3 ±0.90	101.1 ±1.03	100.5 ±1.05	100.2 ±1.04	102.3 ±0.90	101.1 ±1.03	100.5 ±1.05	100.2 ±1.04	102.3 ±0.90	101.1 ±1.03	100.5 ±1.05	100.2 ±1.04
W	101.1 ±0.22	100.4 ±2.37	100.6 ±2.00	99.6 ±1.94 *	102.2 ±2.27	102.1 ±1.92	101.8 ±1.48	101.6 ±1.58	99.8 ±0.20 *a*	100.5 ±0.54 *d*	100.9 ±0.35 *d*	99.5 ±0.72	101.6 ±2.06	100.5 ±1.80	100.3 ±1.69	99.4 ±1.56	99.8 ±0.13 * *a*	98.7 ±0.21 *	98.2 ±0.13	98.0 ±0.21
0.9%	99.9 ±0.48	99.4 ±0.48 *b*	98.6 ±0.18 * *b*	98.3 ±0.27	100.5 ±0.07 * *d*	101.9 ±0.15 *	100.5 ±0.39	99.1 ±0.33	101.2 ±0.32 ** *d*	100.7 ±0.89	99.5 ±0.54	97.9 ±0.12 *b*	101.1 ±0.78	98.9 ±0.86 *b*	97.9 ±0.60 * *b*	98.0 ±0.54 *	99.4 ±0.12 #	98.8 ±0.28 * *b*	98.1 ±0.29 *b*	98.0 ±0.06 *b*
20%	100.0 ±0.49	100.0 ±0.48 *d*	99.3 ±0.06 *d*	98.1 ±0.10 *b*	100.1 ±0.12	99.7 ±0.48 *d*	99.3 ±0.24 *d*	98.9 ±0.15	98.7 ±0.58 #	100.8 ±0.59 *d*	100.1 ±0.83	99.0 ±0.88	99.5 ±0.19 #	100.7 ±0.16 *d*	101.3 ±0.37 *a, b, d*	100.9 ±0.32 *a, b, d*	99.1 ±0.15 # *b*	97.9 ±0.13 #	97.9 ±0.33	97.8 ±0.41
pH of Solution (mean ± SEM; n=3)																				
I	7.69 ±0.01	7.70 ±0.02	7.76 ±0.01	7.74 ±0.00	7.69 ±0.01	7.70 ±0.02	7.76 ±0.01	7.74 ±0.00	7.69 ±0.01	7.70 ±0.02	7.76 ±0.01	7.74 ±0.00	7.69 ±0.01	7.70 ±0.02	7.76 ±0.01	7.74 ±0.00	7.69 ±0.01	7.70 ±0.02	7.76 ±0.01	7.74 ±0.00
W	7.69 ±0.01	7.72 ±0.03 *d*	7.80 ±0.03	7.78 ±0.03	7.74 ±0.01 #	7.78 ±0.02 *d*	7.83 ±0.03	7.81 ±0.02	7.76 ±0.01 #	7.79 ±0.02 *d*	7.85 ±0.02	7.82 ±0.03 #	7.70 ±0.01	7.75 ±0.02 *d*	7.80 ±0.03	7.78 ±0.03	7.67 ±0.02	7.61 ±0.02 #	7.65 ±0.05	7.67 ±0.03
0.9%	7.65 ±0.01	7.52 ±0.03 ^	7.48 ±0.00 ^	7.43 ±0.01 ^	7.47 ±0.04 ^ *a, d*	7.46 ±0.02 ^	7.46 ±0.02 ^	7.36 ±0.01 ^ *a*	7.44 ±0.02 ^ *a, d*	7.45 ±0.02 ^	7.52 ±0.04 ^	7.34 ±0.01 ^ *a*	7.42 ±0.02 ^ *a, d*	7.21 ±0.02 ^ *a,b,c*	7.26 ±0.02 ^ *a,b,c*	7.24 ±0.02 ^ *a,b,c*	7.22 ±0.01 ^ *a*	7.23 ±0.02 ^ *a,b,c*	7.21 ±0.01 ^ *a,b,c*	7.20 ±0.02 ^ *a,b,c*
20%	7.09 ±0.02 ^	7.02 ±0.00 ^	7.04 ±0.01 ^	7.07 ±0.02 ^	7.02 ±0.02 ^	6.95 ±0.01 ^ *a*	6.98 ±0.00 ^ *a*	7.01 ±0.01 ^	6.94 ±0.01 ^ *a, b*	6.98 ±0.02 ^	6.98 ±0.01 ^ *a*	6.98 ±0.01 ^ *a*	6.93 ±0.02 ^ *a, b*	6.96 ±0.01 ^ *a*	6.95 ±0.01 ^ *a, b*	6.91 ±0.01 ^ *e*	6.92 ±0.02 ^ *a, b*	6.94 ±0.00 ^ *a*	6.97 ±0.00 ^ *a, b*	6.94 ±0.00 ^ *a,b,c*

I = in isolation, W = water, 0.9% = 0.9% saline, 20% = 20% saline.

*P<0.05 vs. 20% saline solution; **P<0.03 vs. water and 20% saline solution; #P<0.04 vs. propofol in isolation; ^P<0.05 vs. all other reconstitution solutions.

[a]P<0.03 vs. 10 µg mL^{-1}; [b]P<0.04 vs. 20 µg mL^{-1}; [c]P<0.02 vs. 30 µg mL^{-1}; [d]P<0.04 vs. 50 µg mL^{-1}; [e]P<0.05 vs. all other concentrations

Table 2. pH and percentage of the original remifentanil/propofol remaining over 24 hours when mixed

	10 μg mL^{-1}				20 μg mL^{-1}				30 μg mL^{-1}				40 μg mL^{-1}				50 μg mL^{-1}			
	1 h	**6 h**	**12 h**	**24 h**	**1 h**	**6 h**	**12 h**	**24 h**	**1 h**	**6 h**	**12 h**	**24 h**	**1 h**	**6 h**	**12 h**	**24 h**	**1 h**	**6 h**	**12 h**	**24 h**
Percent Remifentanil Remaining in Remifentanil-Propofol Mixture (mean ± SEM; n=3)																				
W	99.5 ±2.40	81.5 ±1.58	64.7 ±2.49	43.3 ±2.02	100.3 ±3.47	84.4 ±4.41	67.0 ±2.34	46.7 ±2.82	97.9 ±0.72	83.1 ±2.94	67.4 ±3.42	48.2 ±5.09	97.3 ±0.63	81.7 ±1.43 ^	66.0 ±2.56 ^	45.3 ±2.89 ^	97.7 ±0.18	83.1 ±0.53 ^	67.0 ±0.73	46.9 ±1.22
0.9%	93.5 ±0.61	70.5 ±1.24 *	53.8 ±1.15 *	35.0 ±0.49 *d	96.7 ±0.95	74.1 ±1.11 d	58.6 ±1.29 *a, d	35.9 ±1.91 *d	96.0 ±0.49	78.0 ±0.85 a, b	61.2 ±1.36 a	37.4 ±1.34 d	94.4 ±0.74 *	74.9 ±0.98 *a, d	56.7 ±1.19 *d	34.8 ±1.56 *d	95.6 ±0.83	79.7 ±1.01 *a	65.6 ±1.26 a	46.2 ±1.08
20%	96.6 ±2.02	80.8 ±2.08	63.3 ±1.13	45.2 ±2.30	96.0 ±0.43	79.6 ±0.86	66.3 ±0.51	48.9 ±1.93	96.6 ±1.10	83.9 ±0.84 b	71.4 ±0.24 #a, b	53.5 ±0.58 #a	97.5 ±0.36	86.0 ±0.34 b	75.0 ±1.01 a, b, c	56.7 ±0.37 a, b, c	98.8 ±0.22 *	86.8 ±0.67 b	74.8 ±0.80 *a, b, c	60.0 ±1.41 *a, b, c
Percent Propofol Remaining in Remifentanil-Propofol Mixture (mean ± SEM; n=3)																				
W	99.6 ±0.21 c	99.2 ±0.21 d	98.7 ±0.20 b	97.9 ±0.21 c	99.5 ±0.16 c	100.0 ±0.21 d	99.4 ±0.10 ^d	98.3 ±0.38 c	100.6 ±0.00 ^	99.7 ±0.65	99.4 ±0.32 d	99.5 ±0.17 ^	99.6 ±0.19 c	99.3 ±0.40	99.0 ±0.12 d	97.8 ±0.02 c	100.1 ±0.16 c	98.0 ±0.28 #	96.9 ±0.67 #	97.9 ±0.17 c
0.9%	100.8 ±0.62	99.2 ±0.58	98.6 ±0.09	96.8 ±0.97	98.0 ±0.86	99.9 ±0.53	98.0 ±0.65	97.2 ±0.59	98.6 ±0.84	100.5 ±1.80	100.5 ±1.46	99.1 ±1.58	100.1 ±0.26	99.7 ±0.15	98.3 ±0.33	97.7 ±0.06	99.6 ±0.24	99.6 ±0.11	99.3 ±0.49	98.7 ±0.49
20%	99.8 ±0.13	99.4 ±0.62	98.6 ±0.42	99.3 ±0.49	99.0 ±0.27	98.4 ±0.74	97.7 ±0.49	97.3 ±0.79	99.5 ±0.28	98.1 ±0.89	97.9 ±0.84	96.5 ±0.64 a	99.5 ±0.24	99.4 ±0.22	98.4 ±0.35	97.2 ±0.25 a	99.2 ±0.32	98.9 ±0.31	98.5 ±0.46	96.5 ±0.86 a
pH of Solution (mean ± SEM; n=6)																				
W	7.42 ±0.12	7.41 ±0.10	7.42 ±0.12 ^	6.72 ±0.40	7.19 ±0.14 d	7.18 ±0.11	7.12 ±0.13	6.66 ±0.33	7.02 ±0.16	6.98 ±0.13 a	6.95 ±0.16 a	6.55 ±0.36	6.88 ±0.20 a	6.89 ±0.18 a	6.87 ±0.18 a	6.31 ±0.43	6.59 ±0.23 a	6.67 ±0.20 a	6.67 ±0.23 a	6.20 ±0.41
0.9%	7.17 ±0.04	7.17 ±0.05	7.13 ±0.09	6.63 ±0.33	7.10 ±0.08	7.05 ±0.06	6.94 ±0.06	6.64 ±0.26	6.87 ±0.08 a	6.89 ±0.07 a	6.75 ±0.06 a	6.48 ±0.28	6.68 ±0.10 a, b	6.74 ±0.09 a, b	6.66 ±0.10 a, b	6.12 ±0.35	6.44 ±0.16 a, b, c	6.49 ±0.14 a, b, c	6.47 ±0.15 a, b	6.13 ±0.33
20%	7.01 ±0.01 *	7.00 ±0.01 *	6.94 ±0.03	6.36 ±0.31	6.79 ±0.02 *a	6.79 ±0.02 *a	6.69 ±0.08 *, a	5.95 ±0.39	6.64 ±0.02 *a, b	6.66 ±0.01 *a, b	6.62 ±0.03 a	5.84 ±0.38	6.51 ±0.05 e	6.53 ±0.04 e	6.48 ±0.06 a	5.72 ±0.41	6.30 ±0.06 a, b, c	6.34 ±0.06 a, b, c	6.33 ±0.08 a, b, c	5.71 ±0.36

W = water, 0.9% = 0.9% saline, 20% = 20% saline.

*P<0.05 vs. all other reconstitution solutions; #P<0.05 vs. 0.9% saline solution; ^P<0.05 vs. 20% saline solution

[a]P<0.05 vs. 10 μg mL^{-1}; [b]P<0.05 vs. 20 μg mL^{-1}; [c]P<0.05 vs. 30 μg mL^{-1}; [d]P<0.05 vs. 50 μg mL^{-1}; [e]P<0.05 vs. all other concentrations

Figures

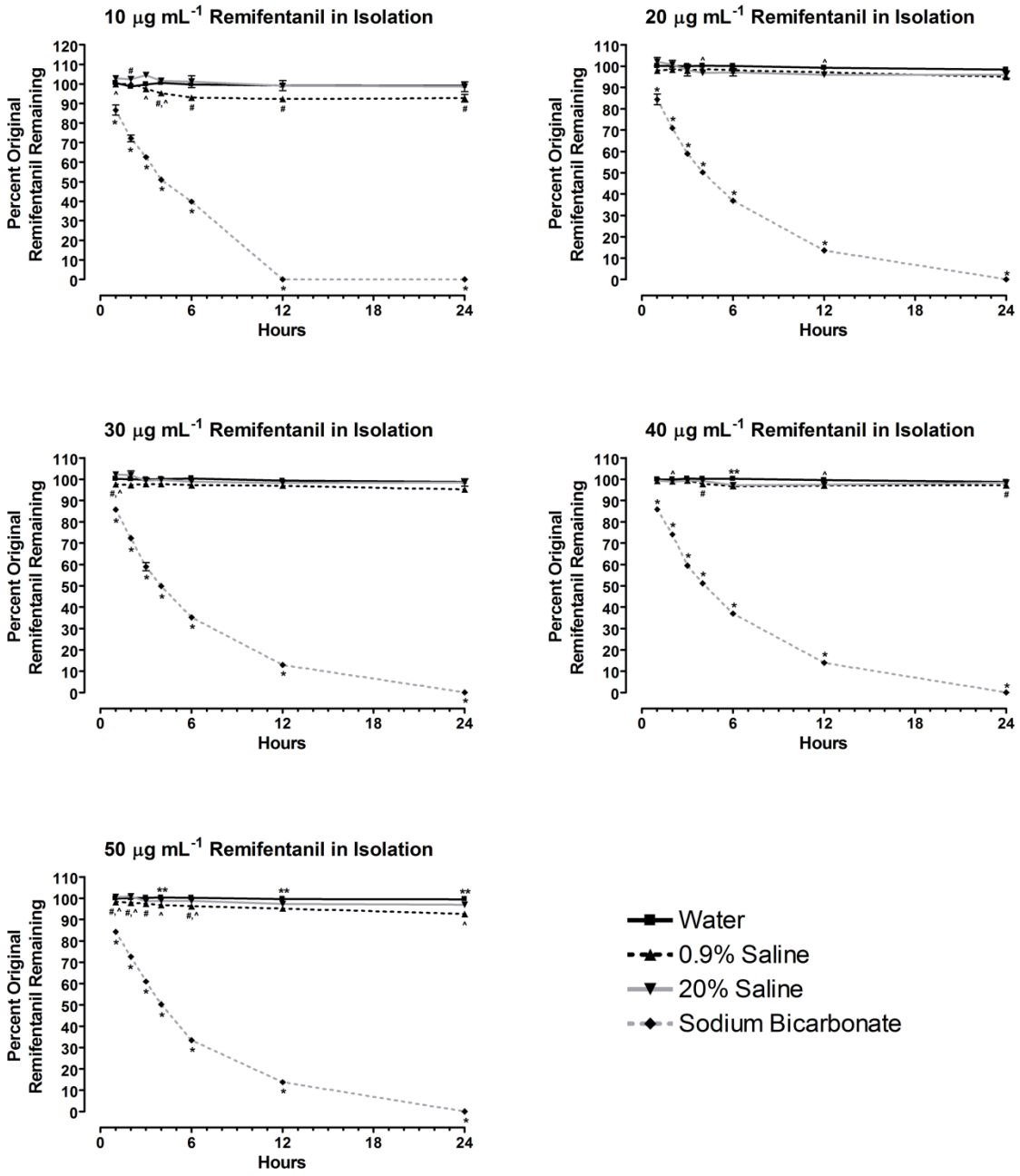

Figure 1. Percent original remaining for 10 μg mL^{-1}, 20 μg mL^{-1}, 30 μg mL^{-1}, 40 μg mL^{-1} and 50 μg mL^{-1} remifentanil reconstituted with water, 0.9% saline, 20% saline and sodium bicarbonate solution and left in isolation over 24 hours. Data expressed as mean ± SEM, n=3. *P<0.01 vs. water, 0.9% saline and 20% saline; $^{\#}$P<0.04 vs. water; $^{\wedge}$P<0.05 vs. 20% saline; **P<0.05 vs. 0.9% saline and 20% saline.

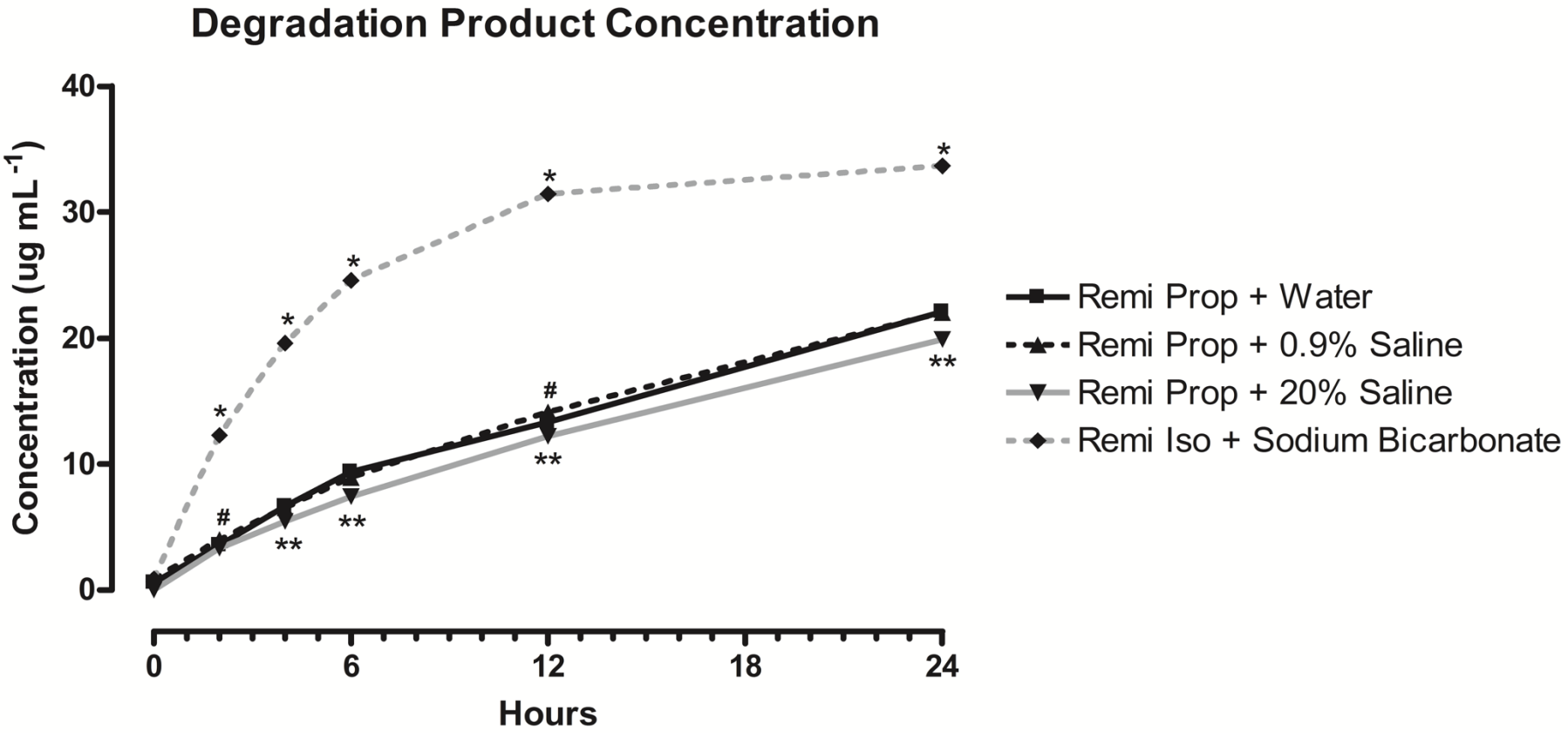

Figure 2. Concentration of the degradation product over 24 hours in samples containing 30 µg mL^{-1} of remifentanil reconstituted with sodium bicarbonate solution in isolation, and in samples containing 30 µg mL^{-1} of remifentanil reconstituted with water, 0.9% saline solution, and 20% saline solution and mixed with propofol. Data expressed as mean ± SEM, n=3. *P<0.05 vs. water, 0.9% saline solution, and 20% saline solution; **P<0.05 vs. water and 0.9% saline solution; #P<0.05 vs. water. "Remi Prop" = remifentanil/propofol mixture; "Remi Iso" = remifentanil in isolation.

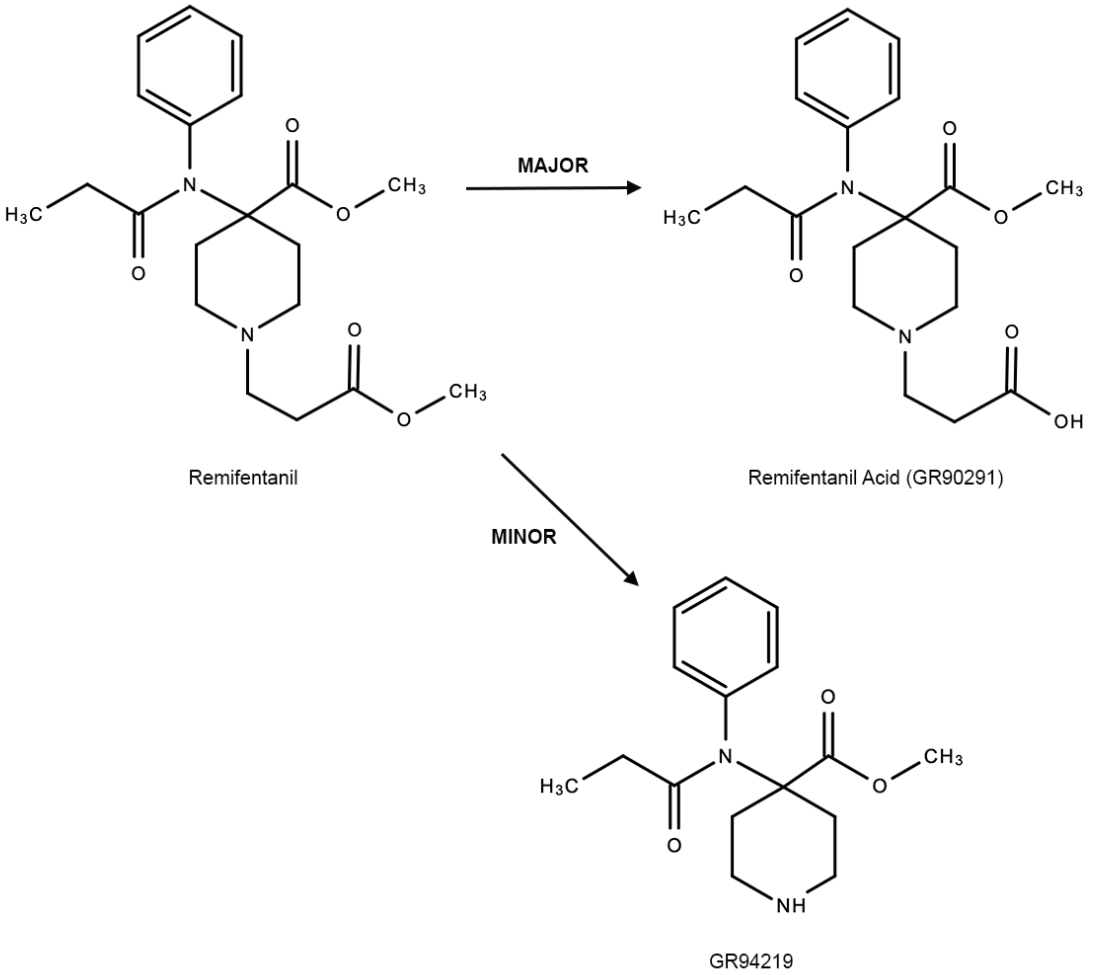

Figure 3. Metabolic pathway of remifentanil showing its major metabolite, remifentanil acid (GR90291), and a minor metabolite (GR94219). Modified from Westmoreland et al. [35]

Additional Table 1. pH of remifentanil over 24 hours when reconstituted with water, saline, or sodium bicarbonate solution

	10 μg mL^{-1}				20 μg mL^{-1}				30 μg mL^{-1}				40 μg mL^{-1}				50 μg mL^{-1}			
	1 h	6 h	12 h	24 h	1 h	6 h	12 h	24 h	1 h	6 h	12 h	24 h	1 h	6 h	12 h	24 h	1 h	6 h	12 h	24 h
	pH of Solution (mean ± SEM; n=3)																			
W	3.67 ±0.04 **	3.69 ±0.02 **, e	3.73 ±0.01 **	3.68 ±0.01 **	4.01 ±0.03 e	3.90 ±0.01 **, e	3.92 ±0.04 ^, e	3.85 ±0.03 **, a	3.86 ±0.02 a, d	3.75 ±0.01 **	3.72 ±0.03 ^	3.77 ±0.01 **, a	3.71 ±0.09 b	3.80 ±0.00 **, b, d	3.82 ±0.00 e	3.82 ±0.04 a	3.48 ±0.06 **	3.63 ±0.01 b	3.58 ±0.01 **, e	3.67 ±0.08
0.9%	4.40 ±0.03 e	4.29 ±0.01 e	4.49 ±0.02 e	4.42 ±0.04 e	4.03 ±0.01 e	3.99 ±0.01 ^, e	3.82 ±0.03 ^	4.16 ±0.02 e	3.92 ±0.01 ^	3.87 ±0.01	3.76 ±0.01 ^	3.97 ±0.04	3.77 ±0.03 b	3.72 ±0.01 b, d	3.87 ±0.01 #, ^, b	3.82 ±0.01 ^, b, d	3.71 ±0.00 ^, b	3.66 ±0.01 ^, b	3.69 ±0.01 ^, e	3.67 ±0.01 ^, b
20%	4.37 ±0.01 e	4.33 ±0.02 e	4.39 ±0.03 e	4.40 ±0.04 e	4.05 ±0.01 e	4.04 ±0.02 e	4.09 ±0.02 e	4.16 ±0.03 e	3.85 ±0.00	3.85 ±0.01	3.89 ±0.01	3.92 ±0.01	3.77 ±0.01 b, d	3.75 ±0.00 b, d	3.81 ±0.01 b, d	3.76 ±0.01 b, d	3.67 ±0.00 b	3.60 ±0.01 b	3.74 ±0.01 b	3.71 ±0.00 b
SB	8.64 ±0.02 *, d	8.62 ±0.03 *	8.79 ±0.02 *, d	8.82 ±0.02 *, d	8.56 ±0.01 *, a, c	8.63 ±0.00 *, b, d	8.73 ±0.03 *, c	8.82 ±0.03 *	8.57 ±0.00 *, a, c	8.64 ±0.00 *, d	8.75 ±0.02 *, c, d	8.85 ±0.02 *, d	8.62 ±0.01 *	8.68 ±0.02 *, d	8.84 ±0.01 *	8.85 ±0.01 *, d	8.55 ±0.01 *, c	8.58 ±0.01 *	8.67 ±0.01 *, c	8.75 ±0.01 *

W = water, 0.9% = 0.9% saline, 20% = 20% saline, SB = sodium bicarbonate.

*P<0.01 vs. water, 0.9% saline solution and 20% saline solution; #P<0.04 vs. water; ^P<0.05 vs. 20% saline solution; **P<0.05 vs. 0.9% saline solution and 20% saline solution

[a]P<0.05 vs. 10 µg mL^{-1}; [b]P<0.05 vs. 30 µg mL^{-1}; [c]P<0.04 vs. 40 µg mL^{-1}; [d]P<0.05 vs. 50 µg mL^{-1}; [e]P<0.05 vs. all other concentrations

3. STUDY B – THE STABILITY OF LYOPHILISED TENECTEPLASE AT HIGH TEMPERATURES

3.1. Chapter summary

Like Chapter 2, this study examines the impact of storage conditions on the stability of a drug (in this instance, tenecteplase) and highlights how controlling the storage environment is crucial for preventing degradation. Temperature, specifically those encountered during transport, is the storage condition of focus for this study, and by simulating realistic scenarios, the findings are highly relevant to emergency service personnel who carry tenecteplase. While stability (through analysis of concentration variations) was the only indication of efficacy in the preceding study, the current study also utilises an *in vitro* model to explore in greater detail how tenecteplase efficacy is affected by modifications to the storage environment. As a supplement to the stability data, this information on clinical functionality further accentuates the importance of controlling the storage conditions of pharmaceuticals, as the direct impact on patient safety is clear.

Tenecteplase is a thrombolytic drug that acts as a plasminogen activator, binding to fibrin on blood clots and converting plasminogen to the enzyme plasmin that lyses the clot. One way it is utilised is for the prehospital treatment of conditions such as AMI and PE by emergency responders. When transported by paramedics, tenecteplase, along with other pharmaceuticals, is stored inside a drug bag, within a response kit, for easy access. As a result, there is a potential for exposure to a variety of environmental conditions, including the high temperatures that are frequently experienced during summer in Central Queensland. Prolonged exposure becomes even more likely in rural or regional areas, as distances travelled can be far and access to the ambulance base may not be as frequent as metropolitan counterparts. This is an issue as many emergency drugs have a suggested storage temperature of no greater than 25°C. If high vehicle temperatures were to impact on tenecteplase efficacy, administration of the correct dose by emergency service personnel may not sufficiently relieve patient symptoms. This would necessitate more thrombolytic to be administered on hospital arrival when the exact amount of efficacious compound already delivered is not known, posing a serious risk of overdose and potentially further increasing the risk of life-threatening haemorrhaging (a possible side effect of thrombolytic therapy). As such, we were interested in developing an experiment that had direct relevance to real-world situations.

The temperature stability of tenecteplase was investigated by subjecting unopened, non-reconstituted vials (i.e. the form in which it is carried by paramedics) to a range of

temperatures over a continuous period of 8 hours; this was deemed a typical shift length and “prolonged” amount of time for a drug carried by emergency responders to be exposed to high temperatures. As the aim of this investigation was to determine tenecteplase stability in excessive temperatures that simulated real-world conditions, the treatments selected were based on a preliminary study we performed that assessed the average and maximum temperatures experienced by a paramedic kit/drug bag left in an ambulance during a Rockhampton summer. This was done by recording the temperature reached inside an ambulance and paramedic drug bag stored within (simulating field conditions), as well as inside a drug bag moved from room temperature and placed on concrete in the sun (simulating roadside conditions). This study was prepared as a brief report, titled “Temperatures reached inside an emergency medical vehicle and paramedic response kit during an Australian summer”, and will soon be submitted for publication; it has been included in this chapter of the thesis, with additional details available in the Appendix (section 7.2.1) of this thesis.

A range of analytical techniques were used to determine tenecteplase degradation. In addition to chemical indications of tenecteplase instability (e.g. through concentration and structural changes), possible physiological effects were also explored *ex vivo*. This was considered an important inclusion as small changes in chemical conformation or stability could result in significantly amplified negative effects in a biological model. Further information on some aspects of this study’s method can also be found in the Appendix (sections 7.2.2 and 7.2.3).

These studies have the potential to impact how tenecteplase and similar pharmaceuticals are transported in emergency vehicles. Not only are the findings of great interest to those who transport tenecteplase in rural or remote areas, but they can also be applied to any location where emergency drugs are subjected to temperatures greater than manufacturers’ suggestions. The anticipated implication of these studies is that they will ensure these important life-saving drugs perform as expected when administered to patients.

The manuscript produced from the main investigation, following the preliminary study brief report in this chapter, has been published in *Prehospital and Disaster Medicine* (the citation can be found in the List of Relevant Publications section of this thesis).

Temperatures reached inside an emergency medical vehicle and paramedic response kit during an Australian summer

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Abstract

Introduction: There are circumstances where emergency service personnel can have infrequent access to their ambulance station. As such, the pharmaceuticals carried in paramedic response kits (PRK) could be unknowingly exposed to temperatures that exceed their recommended storage temperature for prolonged periods, particularly in locations that experience extreme conditions. This has the potential to lead to drug instability and a reduction in pharmacological efficacy. **Study Objective:** By simulating realistic scenarios, the aim of this investigation was to determine how the external environment during an Australian summer impacts upon the maximum temperatures reached inside an emergency vehicle and PRK stored within. **Methods:** This study was conducted during summer in a regional Australian city. Temperatures were recorded inside an emergency vehicle and paramedic response kit when the vehicle was parked in the sun (simulating field conditions) or inside a storage shed for 9-hour intervals, and when the kit was removed from the vehicle and placed in the sun (simulating roadside conditions) for two 1-hour periods: when the increase in external temperature was at a maximum, and when the highest external temperature was reached. **Results:** When parked in the sun, a maximum vehicle temperature of 45.8°C was recorded, while PRK temperatures reached 43.8°C and had a mean kinetic temperature (MKT) of 37.5°C. Inside the storage shed, temperatures reached 37.9°C inside the vehicle and 36.4°C in the PRK, with an MKT of 32.9°C. When the PRK was removed from room temperature and placed in the sun for two 1-hour periods, the temperature within increased 8.7°C and 7.6°C, respectively, and reached an average maximum of 36.5°C for both sessions (MKT of 32.4°C and 32.9°C). In all scenarios, temperatures inside the kit increased rapidly and continued to rise, even when vehicle temperatures had decreased. **Conclusion:** Drugs kept inside the PRK can be unknowingly subjected to very high temperatures, possibly leading to instability, degradation, or loss of pharmacological efficacy. Proper management of pharmaceutical storage conditions in emergency vehicles is crucial for ensuring clinical efficacy and patient safety are preserved.

Introduction

Drug stability incorporates physical, chemical, microbiological, therapeutic, and toxicological characteristics. Environmental conditions have a significant impact on drug stability and encompass factors such as temperature, humidity, and light.[1]

Most emergency pharmaceuticals carried by paramedics require storage at controlled room temperature, defined by the United States Pharmacopeia as 20 – 25°C (68 – 77°F), while the mean kinetic temperature (MKT) is not to exceed 25°C.[2] Drugs can have mixed tolerability to high temperature,[3] but adhering to recommended storage conditions ensures full drug stability and clinical efficacy is retained. As there are circumstances where emergency medical personnel can have infrequent access to their base, such as those servicing rural or remote areas, drugs they transport may be kept in a paramedic response kit (PRK) within the emergency vehicle for extended periods. Maintaining ideal storage conditions in these circumstances, particularly in extreme temperatures, can be difficult.[4] Improper storage resulting in drug instability may produce adverse effects, such as a decrease in pharmacological efficacy, safety, ease-of-use, and patient acceptability, and the development of toxic degradation products.[1]

Several studies conducted in the US, Europe, and South Africa have identified that out-of-hospital medications are regularly exposed to temperatures exceeding 25°C (to various extents, depending on location) during transit. However, the majority of these previous investigations only took temperature readings within either the drug storage compartment or vehicle (rarely both), and to our knowledge, none have considered the increase in temperature inside drug bags when placed outside the vehicle for a period of time to simulate attending to patients roadside, nor have any been conducted under Australian summer conditions.[3,5-9] Recording temperatures within the drug bag and vehicle concurrently would indicate the time it takes for changes in vehicle temperature to affect the drug bag stored within, while analyzing the temperature inside a drug bag removed from the vehicle is an important aspect of an emergency drug's treatment in the field that is currently lacking.

Therefore, the objective of this study is to determine how the external environment can impact upon the maximum temperatures reached inside an emergency vehicle and the PRK stored within, and in the PRK when it is removed from the vehicle and placed outside, during the height of an Australian summer. This will indicate the unfavorable conditions pharmaceuticals may be exposed to in similar settings worldwide.

Methods

Measurements were taken during the summer of 2018 in the regional Australian city of Rockhampton, Queensland, using a LabQuest interface with temperature probes attached (Vernier Software & Technology, Beaverton, OR, USA). The emergency vehicle (2012 Sprinter Van, Mercedes-Benz, Stuttgart, Germany) and PRK (NEANN brand, RAPP Australia Pty Ltd, Lara, VIC, Australia) used throughout the study, equivalent to those utilized by Queensland Ambulance Service, were kindly on loan from CQUniversity Australia (Rockhampton, QLD, Australia). All recordings were performed in triplicate and external temperatures were obtained from the Australian Bureau of Meteorology.[10]

Vehicle and PRK temperature measurements

One temperature probe was placed on the floor of the patient treatment area of the vehicle, while another was placed inside the drug bag attached to the PRK that was stored inside the vehicle, near the door. The vehicle, with windows and doors closed, was parked on concrete, either in the sun (simulating conditions in the field) or inside a storage shed, and temperatures were recorded every half-hour over a 9-hour period (9 am – 6 pm).

PRK-only temperature measurements

A temperature probe was placed in the drug bag attached to the PRK. The PRK was then moved from room temperature and placed on concrete in the sun (simulating roadside conditions) for two 1-hour periods a day: when the increase in external temperature was maximum (between 10 and 11 am); and when the highest external temperature was reached (between 2 and 3 pm). The temperature was recorded every 2 minutes and the PRK was returned to room temperature following each hour-long session.

Statistical analysis

ANOVA was performed on each treatment group (i.e. vehicle in sun, vehicle in shed, and PRK in sun) using a significance value of 0.05. Where statistical significance was detected, Student's *t* test was applied on each timepoint (also using a significance value of 0.05) between the following groups: external temperature vs. vehicle temperature, vehicle temperature vs. PRK temperature (for "Vehicle and PRK temperature measurements"), and external temperature vs. PRK temperature (for "PRK-only temperature measurements") (Prism 4.02; GraphPad Software, San Diego, CA, USA).

Results

Vehicle and PRK

When parked in the sun, a maximum average external temperature of 40.5°C (95% CI, 39.5–41.5) was recorded. The temperature inside the emergency vehicle reached a maximum average of 45.8°C (95% CI, 45.2–46.4), maintaining temperatures over 45°C for 2.5 hours (Figure 1), and significantly exceeded external temperatures after 2.5 hours ($P<0.0169$). The PRK within the vehicle reached a maximum average of 43.8°C (95% CI, 43.0–44.6) and had an MKT of 37.5°C. PRK temperatures exceeded external temperatures after 5 hours, and after 7 hours, differences between PRK and vehicle temperatures were no longer significant (Figure 1).

When the vehicle was parked inside a storage shed, external temperatures reached a maximum average of 35.5°C (95% CI, 30.6–40.4). Vehicle temperatures reached 37.9°C (95% CI, 33.7–42.1), exceeding external after 3.5 hours (Figure 1). PRK temperatures had a maximum average of 36.4°C (95% CI, 32.8–40.0; MKT of 32.9°C) and exceeded external temperatures after 7 hours (Figure 1).

PRK only

In the 1-hour intervals when the increase in external temperature was maximum (average temperature of 32.9°C; 95% CI, 31.7–34.1) and the highest external temperature was attained (average temperature of 34.1°C; 95% CI, 33.1–35.1), the temperature in the PRK increased an average of 8.7°C (31.3%; 95% CI, 1.34–16.1; MKT of 32.5°C) and 7.6°C (26.3%; 95% CI, 4.09–11.1; MKT of 32.9°C), respectively (Figure 2). A maximum average temperature of 36.5°C (95% CI, 32.6–40.4) was reached within the PRK during both sessions. During the morning session, the temperature in the PRK exceeded the external temperature after 33 minutes, and significantly exceeded its initial temperature after 34 minutes ($P<0.0471$). In the afternoon, the PRK temperature exceeded external after 44 minutes, and significantly exceeded its initial temperature after 30 minutes ($P<0.0500$).

Discussion

Emergency medical personnel may be required to travel great distances that do not facilitate frequent access to their base, where appropriate drug storage facilities (such as refrigeration) are usually available. Through simulation of realistic scenarios faced by emergency service workers (particularly those in regions in Australia that experience extreme temperatures), the aim of this study was to determine how external conditions can affect the storage temperature of the medications they transport. This is an important area of research as prolonged exposure to high temperatures has the potential to cause drug instability and decrease efficacy.[1]

As seen in previous studies, temperatures reached inside the vehicle eventually exceeded the external temperature.[7] External conditions were found to affect vehicle temperatures more rapidly than PRK temperatures. Not only was the rate of temperature increase inside the vehicle greater than within the PRK, but vehicle temperatures also responded to decreases in the external temperature more quickly. However, this resulted in PRK temperatures continuing to increase after external and vehicle temperatures had decreased, both in the sun and in the storage shed. When parked in the sun, PRK temperatures increased 12.8°C (41.2%) over the 9-hour period, while in the storage shed they increased 6.7°C (22.6%) over the same period. For emergency vehicles that are required to be in the field for hours, these findings show that, without proper management, high external temperatures can have an extensive and enduring impact on temperatures within the PRK. This is noteworthy as PRK temperatures were found to greatly exceed the generally recommended storage MKT of 25°C, regardless of vehicle location.

A unique aspect of the current study was removing the PRK from room temperature and placing it on concrete in the sun to simulate roadside patient attendance. While doing this showed comparable effects on PRK temperatures compared to storage inside the vehicle (and corroborated with findings from Palmer et al. that temperatures inside a drug box can exceed outside ambient air temperature[11]), without the vehicle the temperature increases within the PRK were more extreme and occurred in a shorter timeframe. Within an hour, the internal temperature increased an average of 8.2°C (28.7%); this was greater than the increase seen over 9 hours for the PRK stored in the vehicle parked in the shed. Furthermore, the average time taken for the PRK temperature to exceed the external temperature was only 38.5 minutes. This demonstrates that placing the PRK roadside for even a short period can result in a large increase in the internal temperature. If the difference between the initial internal and external temperatures had been greater (i.e. if the PRK had been stored in cooler, air-conditioned conditions, such as a running vehicle),

the rate of temperature increase would have been more pronounced. Based on the earlier findings, it is also speculated that the internal PRK temperature would continue increasing after being returned to the vehicle. Therefore, the impact on medications stored within would be compounding. This makes awareness of external heat effects on PRK temperatures a crucial aspect of emergency drug storage.

The PRK and drug bag used in this study were constructed of a padded, coated material. Intended to be durable and protective, it inevitably provides insulation that was found to be unfavorable in hot climates. Depending on the material used for construction, these insulating properties may not be present in all PRKs. However, the PRK in this study is currently used by a large emergency care provider in Australia,[12] making the findings extremely applicable. It is suggested that subsequent studies investigate the impact of heat on the efficacy of emergency drugs in their original, packaged form. This would provide emergency medical personnel with highly relevant data on how uncontrolled temperature can impact upon the pharmaceuticals they carry, and may result in modifications to drug storage during transport.

While this study was modelled in remote areas where access to an ambulance station may be minimal, temperature fluctuations within the PRK were extreme and occurred quickly. Therefore, it is likely that similar outcomes would be encountered in more urban locations. High temperature exposure is an important consideration for emergency medical personnel as it is known to negatively impact on the efficacy of certain drugs, including some lyophilized protein formulations.[13] It is hoped that by increasing awareness of how PRK temperatures are impacted by heat, emergency service providers will be more vigilant in ensuring storage conditions are properly managed so that the clinical efficacy of the pharmaceuticals they transport is preserved.

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Figures

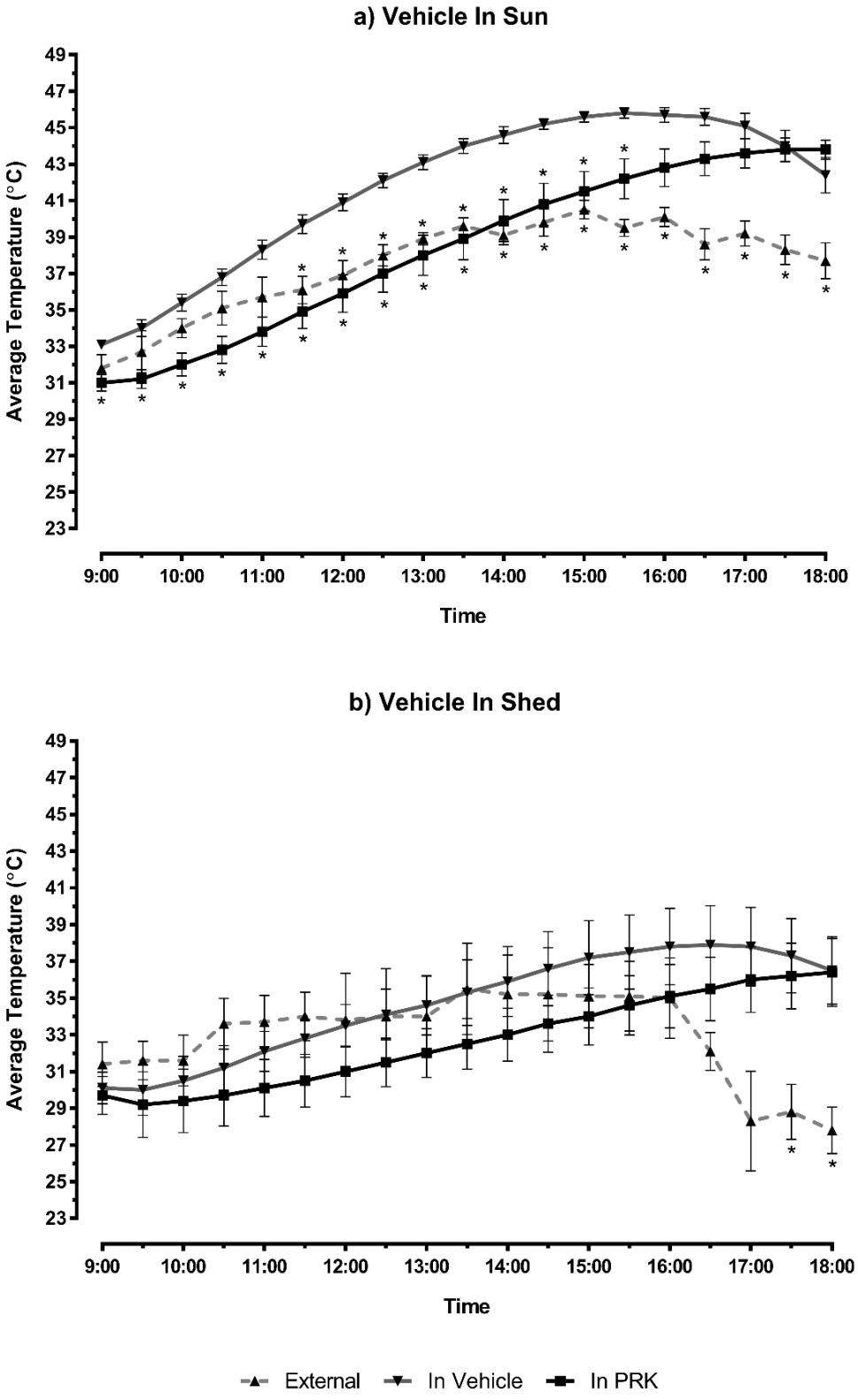

Fig. 1. Average temperature reached outside, within an emergency service vehicle, and within a paramedic response kit (PRK) stored inside the vehicle, when a) parked on concrete and in the sun, and b) in a storage shed, during summer. Data expressed as mean ± SD, n=3; statistical analysis performed using one-way ANOVA and Student's *t* test. *P<0.05 vs. temperature in vehicle.

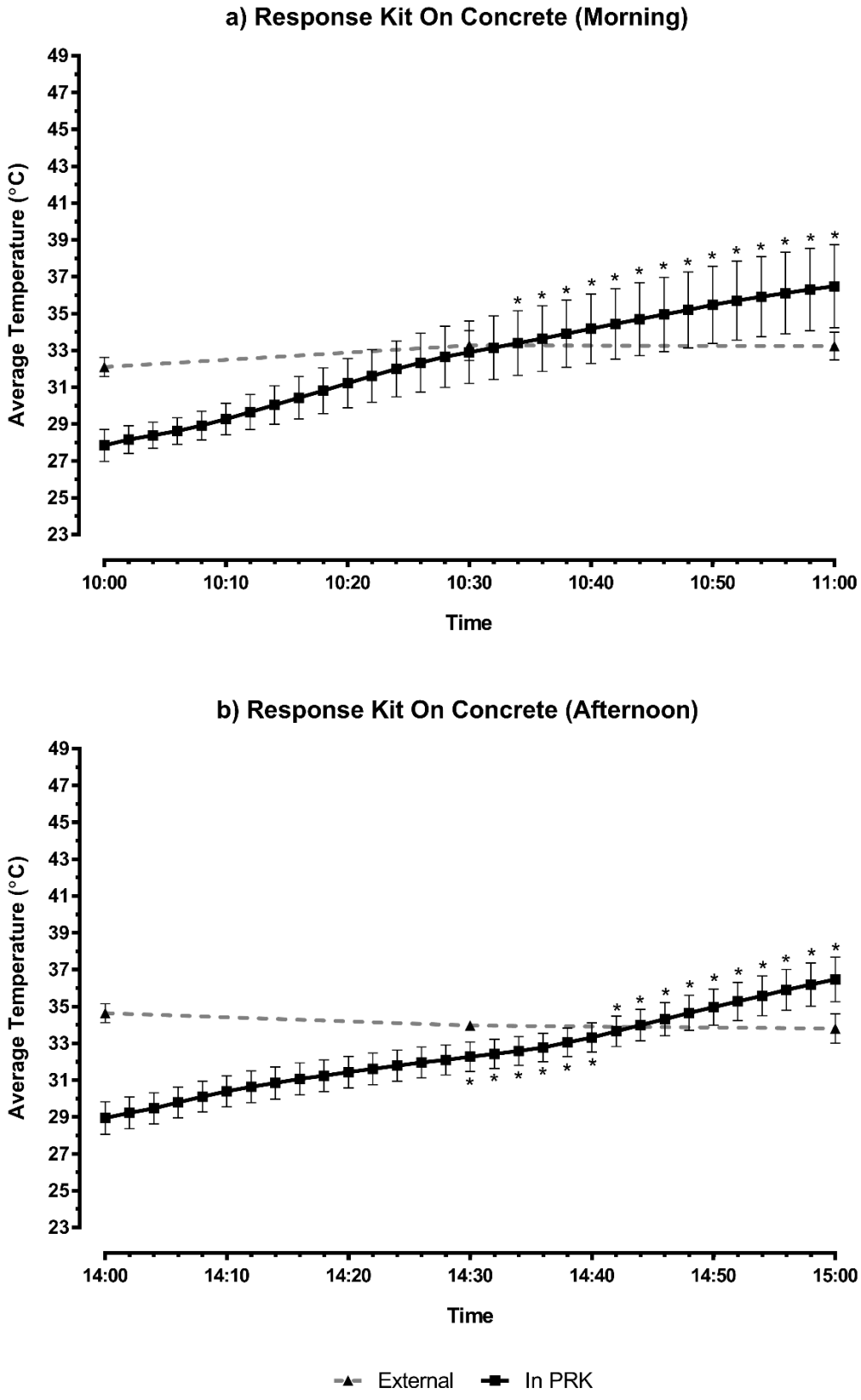

Fig. 2. Average temperature reached outside and within a paramedic response kit (PRK) removed from room temperature and placed on concrete, in the sun, for an hour when (a) the change in external temperature was the greatest, and (b) the external temperature was maximum. Data expressed as mean ± SD, n=3; statistical analysis performed using Student's *t* test. *P<0.05 vs. initial PRK temperature.

The Effect of High Storage Temperature on the Stability and Efficacy of Lyophilized Tenecteplase

Short running title: Heat Effects on Lyophilized Tenecteplase

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Keywords: drug stability; drug storage; Emergency Medical Services; prehospital emergency care; tenecteplase

Abbreviations:

AMI: acute myocardial infarction

DAD: diode-array detection

DTT: dithiothreitol

ESV: emergency service vehicle

FEU: fibrinogen equivalent units

HPLC: high-performance liquid chromatography

MI: myocardial infarction

PE: pulmonary embolism

SDS: sodium dodecyl sulfate

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Abstract

Introduction: Tenecteplase is a thrombolytic protein drug used by paramedics, emergency responders, and critical care medical personnel for the prehospital treatment of blood clotting diseases. Minimizing the time between symptom onset and the initiation of thrombolytic treatment is important for reducing mortality and improving patient outcomes. However, the structure of protein drug molecules makes them susceptible to physical and chemical degradation that could potentially result in considerable adverse effects. In locations that experience extreme temperatures, lyophilized tenecteplase transported in emergency service vehicles (ESVs) may be subjected to conditions that exceed the manufacturer's recommendations, particularly when access to the ambulance station is limited. **Study Objective:** This study evaluated the impact of heat exposure (based on temperatures experienced in an emergency vehicle during summer in a regional Australian city) on the stability and efficacy of lyophilized tenecteplase. **Methods:** Vials containing 50mg lyophilized tenecteplase were stored at 4.0°C (39.2°F), 35.5°C (95.9°F), or 44.9°C (112.8°F) for a continuous period of eight hours prior to reconstitution. Stability and efficacy were determined through assessment of: optical clarity and pH; analyte concentration using UV spectrometry; percent protein monomer and single chain protein using size-exclusion chromatography; and *in vitro* bioactivity using whole blood clot weight and fibrin degradation product (D-dimer) development. **Results:** Heat treatment, particularly at 44.9°C, was found to have the greatest impact on tenecteplase solubility; the amount of protein monomer and single chain protein lost (suggesting structural vulnerability); and the capacity for clot lysis in the form of decreased D-dimer production. Meanwhile, storage at 4.0°C preserved tenecteplase stability and *in vitro* bioactivity. **Conclusion:** The findings indicate that, in its lyophilized form, even relatively short exposure to high temperature can negatively affect tenecteplase stability and pharmacological efficacy. It is therefore important that measures are implemented to ensure the storage temperature is kept below 30.0°C (86.0°F), as recommended by manufacturers, and that repeated refrigeration-heat cycling is avoided. This will ensure drug administration provides more replicable thrombolysis upon reaching critical care facilities.

Introduction

Tenecteplase is a recombinant tissue-type plasminogen activator that elicits its effects by converting plasminogen to plasmin, degrading fibrin in blood clots to produce measurable fibrin degradation products (D-dimer being the primary enzymatic degradation product).[1,2,32] Developed via modifications to three amino acid sites of another plasminogen activator, alteplase, tenecteplase has the benefits of increased fibrin specificity, greater resistance to plasminogen activator inhibitor PAI-1, and a longer plasma half-life.[3] With the additional advantage of a decreased risk of major bleeding, tenecteplase is an effective treatment for acute myocardial infarction (AMI) and pulmonary embolism (PE).[4]

Fibrinolytic therapy as a method of reducing mortality of myocardial infarction (MI) is considerably more effective when the time between symptom onset and commencement of treatment is minimalized.[5] In large metropolitan centers with multiple specialized cardiac catheterization emergency facilities, thrombolysis is initiated in the hospital setting. However, in areas with long transit times (eg, rural settings), initiating treatment prior to hospital arrival through advanced or intensive care paramedic specialist intervention is believed to reduce both the time to treatment and total mortality, resulting in improved patient outcomes compared to those first treated in hospital.[6-9]

While non-protein drugs can be affected by thermolysis (eg, diazepam and lorazepam – benzodiazepines used for prehospital seizure treatment), protein drug molecules (including tenecteplase, insulin, and various monoclonal antibodies [mAbs]) have a structural complexity that predisposes them to physical and chemical degradation via environmental and solution conditions during manufacturing, storage, shipment, and patient administration.[10-14] Maintaining the overall structural configuration is essential in ensuring a therapeutic protein's stability, efficacy, safety, and immunogenicity, with only minor structural changes potentially resulting in major negative impacts.[15] For example, disruption of the high order structure that results in decreases in both protein monomer and single chain protein can affect a drug's selectivity, potentially reducing efficacy and inducing adverse side effects.[3,16] Any structural alterations are sometimes only identified during long-term or accelerated storage involving deviations to the surrounding environment (eg, temperature fluctuations).[13]

To limit accelerated thermal degradation of medications, it is suggested they are stored at controlled room temperature unless otherwise indicated.[17] Specifically, the manufacturers of Metalyse, a brand of tenecteplase marketed by Boehringer Ingelheim Pty Limited (North Ryde, NSW, Australia), recommend the lyophilized form be stored below 30.0°C (86.0°F).

Several studies have demonstrated the difficulty associated with ensuring proper storage of pharmaceuticals in emergency service vehicles (ESVs), particularly in areas that experience extreme temperatures.[18] This is even more challenging in rural areas, where limited access to temperature-controlled storage facilities could potentially result in prolonged subjection to less-than-ideal conditions. This is because, in these circumstances, emergency medical personnel are required to store the insulated drug bag containing all of their necessary medication in a designated area within their vehicle for the entire shift, for numerous shifts, with the vehicle air-conditioning system being the only source of cooling (R Jackson 2018; personal communication). In a regional city in Queensland, Australia, a preliminary investigation found that the mean temperature inside a drug bag stored in an ESV parked outside during the summer months was 35.5°C (95.9°F), with a maximum of 44.9°C (112.8°F) reached (unpublished findings). These temperatures far exceed those suggested for tenecteplase storage. While the stability thresholds provided by pharmaceutical manufacturers are minimums,[18] the extent to which these thresholds can be exceeded for each specific pharmaceutical transported in ESVs is not fully understood. The result of this potential decrease in efficacy of tenecteplase due to field transportation and delivery has resulted in some variable clinical outcomes, with potential incomplete clot thrombolysis and resolution of altered electrocardiogram traces reported by advanced care paramedics and an emergency treating cardiologist (2017; personal communication).

The effect of storing reconstituted tenecteplase at high temperature (i.e. 37°C) for 24 – 96 hours was previously explored by Lentz, Joyce and Lam, who found there was no change in tenecteplase concentration following exposure.[16] However, as manufacturers recommend tenecteplase be used immediately following reconstitution due to concerns with possible microbiological growth (or otherwise stored at 2.0°C-8.0°C [35.6°F-46.4°F] for no longer than 24 hours), it is transported and stored in its lyophilized form. Despite the relevance to paramedics and clinicians, studies investigating the impact of storing tenecteplase at high temperatures prior to reconstitution are limited. Therefore, the aim of the present investigation was to determine how the stability and efficacy of lyophilized tenecteplase was affected after being stored for eight hours at temperatures exceeding those suggested by its manufacturers. The environmental conditions examined in this study simulated those experienced by ESVs during summer months in a regional city in Queensland, Australia, but are directly applicable to similar use and climates encountered world-wide.

Methods

Tenecteplase (Metalyse), containing 50mg active ingredient, 522mg arginine, phosphoric acid adjusted to pH 7.3, and 4.0mg polysorbate-20, was obtained from the Queensland Department of Health Central Pharmacy (Richlands, Queensland, Australia) and stored at 4°C (39.2°F) until study commencement. L-arginine free base, 1,2-propanediol, sodium phosphate, sodium dodecyl sulfate (SDS), urea, and dithiothreitol (DTT) were purchased from Astral Scientific Pty Ltd (Taren Point, New South Wales, Australia). Polysorbate-20 and Tris were purchased from Chem- Supply Pty Ltd (Port Adelaide, Southern Australia, Australia). Milli-Q water was prepared by an ultrapure water system (Merck Millipore; Bayswater, Victoria, Australia).

Heat Treatment

Duplicate vials containing 50mg lyophilized tenecteplase, protected from light in their original packaging, were stored at either 4.0°C, 35.5°C (average temperature reached inside a paramedic drug bag stored in an emergency vehicle during summer, as determined previously by the authors; results unpublished), or 44.9°C (maximum temperature reached within a drug bag stored in an emergency vehicle, also determined previously by the authors; results unpublished) for eight hours. Following treatment, each vial was reconstituted with water for injection that was packaged with the drug to a final concentration of 5mg/mL, as per product preparation instructions. Aliquots were removed from each vial and used in subsequent assays, as previously described by Semba, et al and Lentz, Joyce, and Lam,[16,19] to determine tenecteplase stability and efficacy.

Assays

Optical Clarity and pH—Solutions were visually inspected against a white and black background for any obvious precipitate formation. pH was determined using a Eutech Instruments pH 700 meter (Thermo Fisher Scientific; Singapore).

Concentration—Aliquots were diluted 50-fold in a buffer containing 300mM arginine phosphate and 0.04% polysorbate-20, pH 7.3, and assayed using a Genesys 10S UV-Vis spectrometer (Thermo Scientific; Madison, Wisconsin USA) at 280nm. Absorbance at 320nm was recorded to correct for light scattering. Concentration was calculated using the Beer-Lambert equation with an extinction coefficient of 1.9mL/mg/cm (absorptivity of tenecteplase at 280nm), as follows:

$$\text{Conc. (mg/mL)} = \frac{\text{abs. @ 280 nm - abs. @ 320 nm}}{1.9}$$

Protein Monomer—Samples (25µg tenecteplase) were analysed using size exclusion chromatography on a Tosoh Bioscience TSKgel G3000SWxl column (300x7.8mm; 5µm) (Minato-ku; Tokyo, Japan) employed on an Agilent Technologies 1200 series high-performance liquid chromatography system (HPLC; Melbourne, Victoria, Australia) with diode-array detection (DAD) at 280nm. The mobile phase consisted of 200mM arginine free base, 150mM Tris, and 5% 1,2-propanediol, pH 7.0, at a flow rate of 1.0mL/min. The percent monomer in each sample was calculated as follows:

$$\% \text{ monomer} = \frac{\text{monomer peak area}}{\text{total protein peak area}} \times 100$$

Single Chain Protein—Aliquots were diluted in a buffer of 8M urea solution and 200mM sodium phosphate, pH 6.8, and reduced for three to five minutes with 20mMDTT solution (final concentration) at 37.0°C (98.6°F). Analysis of samples (12.5µg tenecteplase) was performed on a Tosoh Bioscience TSKgel G3000SWxl size exclusion column (300x7.8mm; 5µm) employed on an Agilent Technologies 1200 series HPLC with DAD at 214nm. The mobile phase contained 200mM sodium phosphate and 0.1% SDS, pH 6.8, at a flow rate of 1.0mL/min. The percent single chain was calculated as follows:

$$\% \text{ single chain} = \frac{\text{first main peak area}}{\text{sum of 1st and 2nd peak areas}} \times 100$$

Clot Weight—This assessment was based on a method proposed by Prasad, et al.[20] Briefly, healthy human venous blood was transferred to pre-weighed microcentrifuge tubes and incubated at 37.0°C for 45 minutes to allow for clot formation. Following serum removal, clots were weighed using a Mettler Toledo AB204-S balance (Greifensee, Switzerland). Aliquots of 100µL treated tenecteplase (ie, those samples exposed to various temperatures; nominal concentration of 5mg/mL) were added to the clots, with water used as a negative control. After further incubation at 37.0°C for 90 minutes, excess fluid was removed and retained, and the tubes weighed once more to compare to initial clot weight.

Fibrin Degradation Product—The serum from all samples collected before and after treatment was analyzed for the presence of D-dimer using the INNOVANCE D-Dimer immunoturbidimetric assay on a Sysmex CA-560 Coagulation Analyzer (Siemens Healthcare Pty Ltd; Bayswater, Victoria, Australia).

Statistical Analysis

Statistical analysis was performed on all data using ANOVA, with Student's *t* test applied

when significance (P <.05) was identified (Prism version 4.02; GraphPad Software; San Diego, California USA).

Results

Optical Clarity and pH

Tenecteplase stored at 4.0°C reconstituted easily, however, there was some difficulty experienced with fully reconstituting vials stored at 35.5°C and 44.9°C. Particulate matter was temporarily observed in these solutions following diluent addition, indicating the drug had not completely dissolved. Full reconstitution had visually occurred by the time analyses were conducted.

The pH of reconstituted solutions was found to be similar for all temperatures tested (Table 1).

Concentration

Tenecteplase concentration was not significantly different between temperature treatments after eight hours (Table 1). There was a difference of 0.01mg/mL (10.8%) between 4.0°C and 35.5°C vials and 0.02mg/mL (20.0%) between 4.0°C and 44.9°C vials.

Protein Monomer

Minimal loss of protein monomer was observed in lyophilized tenecteplase stored at 4.0°C and 35.5°C with an average of 98.4% (95% CI, 97.9-98.9) and 98.6% (95% CI, 98.4-98.7) protein monomer detected after eight hours, respectively. There was a greater decrease in protein monomer for vials stored at 44.9°C, averaging 97.7% (95% CI, 97.3-98.0) after eight hours (Figure 1).

Single Chain Protein

As storage temperature increased, the amount of single chain protein detected decreased. Vials stored at 4.0°C contained an average of 37.5% (95% CI, 34.7-40.3) of the single chain form after eight hours, while this percentage averaged 31.6% (95% CI, 27.4-35.8) and 29.7% (95% CI, 29.1-30.3) in solutions stored at 35.5°C and 44.9°C, respectively (Figure 1).

Clot Weight

All tenecteplase solutions were found to elicit some clot lytic activity following eight hours of temperature exposure. Interestingly, the control (water-treated) solutions showed more variance in clot weight than those treated with tenecteplase with an average decrease of 0.0267g (95% CI, 0.0207-0.0326) or 9.3%. Tenecteplase stored at 4.0°C and 35.5°C caused a similar reduction in clot weight with an average decrease of 0.0130g, or 4.8%

(95% CI, 0.0101-0.0159) and 4.8% (95% CI, 0.00648-0.0196), respectively. Solutions stored at 44.9°C produced the smallest decrease in clot weight compared to all other treatments, averaging a decrease of 0.0115g (95% CI, 0.00288-0.0201) or 4.4% (Figure 2).

Fibrin Degradation Product

For all samples analyzed, D-dimer levels increased following water/thrombolytic treatment compared to initial levels. However, this change was considerably less for control samples (an average increase of 0.50mg/L Fibrinogen Equivalent Units [FEU] (95% CI, 0.45-0.54) or 109.1%) compared to those treated with tenecteplase. Samples stored at 4.0°C had the greatest elevation in D-dimer, increasing an average of 34.55mg/L FEU (95% CI, 34.46-34.63) or 7668.0%, followed by samples stored at 44.9°C (average increase of 27.75mg/L FEU (95% CI, 14.60-40.90) or 5429.0%); these increases were significantly greater than that observed with control samples ($P < .04$). The amount of D-dimer in samples stored at 35.5°C was the least of all tenecteplase solutions, increasing an average of 23.87mg/L FEU (95% CI, 3.20-44.53) or 4222.0% following treatment (Figure 2).

Discussion

This investigation explored the effect of storing lyophilized tenecteplase at temperatures exceeding those suggested by manufacturers over an eight-hour period. A number of factors, as previously described, were evaluated to assess the drug's stability and efficacy following heat treatment with the temperatures chosen simulating those that occur in ESVs in a regional city in Queensland, Australia.[16,19,20] A unique aspect of this study was that tenecteplase was stored in its original, lyophilized form; similar studies typically store the drug in its reconstituted form. Lyophilization, or the freezing and drying of products, is a technique commonly used to increase the physical and chemical stability of labile therapeutic proteins by hindering degradation reactions.[21] Exploring tenecteplase stability in its lyophilized form was deemed an important inclusion in this study because of its applicability to real-world emergency situations.

When stored at 35.5°C or 44.9°C for eight hours, tenecteplase concentration was not significantly affected. While this investigation was conducted over one continuous eight-hour period to represent an extreme situation, it is likely that the drugs would be subjected to shorter (eg, two or three hours), more frequent cycles of refrigeration and reheating as the drugs are moved from temperature-controlled storage to the warm vehicle, and vice versa, between call-outs. The accumulated effects of this could result in continued degradation of the product. It is also important to highlight the temporary reduction in solubility of the drugs stored at the higher temperatures compared to those stored under refrigeration, as this presents potential complications for emergency medical personnel in the field. In situations where a patient requires emergency treatment, a solution may not be given adequate time to dissolve fully. This could lead to a reduction in the lysis capacity of the drug through decreased concentration or deactivation of the effective constituent, or could result in precipitation of harmful chemical entities.[22] Because of the importance of ensuring no particulates are present in the reconstituted solution, it is suggested that, regardless of solution stability once fully reconstituted, prolonged exposure of lyophilized tenecteplase to high temperatures should be avoided.

Other indications of drug instability were observed. The amount of single chain protein present decreased as storage temperature increased, and the greatest loss in protein monomer was found in solutions stored at the highest temperature. Loss of protein monomer indicates fragmentation of the protein and possible disruption of its high order structure, potentially increasing susceptibility to aggregation and subsequent antibody-mediated adverse immune responses.[16,19,23] The conversion of tenecteplase from its one-chain to two-chain form occurs as a result of proteolytic cleavage or hydrolytic clipping at

Arg 275-Ile 276 on contact with plasmin.[16,24,25] A decrease in protein one-chain is indicative of reduced fibrin specificity; although both the one- and two-chain forms of tenecteplase exhibit thrombolytic activity, it is the one-chain form that is more fibrin-specific.[26] The fibrin specificity of tenecteplase provides such clinical benefits as better dissolution of older fibrin clots and fewer non-cerebral bleeding complications.[3] Therefore, the findings that indicate storing tenecteplase at high temperatures could result in the breakdown of the protein structure may also suggest a decrease in its fibrin specificity and the associated benefits of minimal systemic plasminogen activation and fibrinogen depletion.[27]

The *in vitro* whole blood clot model for determining tenecteplase bioactivity was used for this study as Prasad, et al had successfully demonstrated the clot lysis activity of another thrombolytic medication, streptokinase, using the same method.[20] In the current study, solutions stored at 4.0°C and 35.5°C showed similar clot lysis ability while 44.9°C solutions exhibited less. This would suggest that storage at high temperatures negatively affects tenecteplase efficacy. However, control solutions were found to exhibit the greatest clot lysis ability of all treatments tested. There are several factors that can affect thrombolysis, including the time allowed for clot formation and/or clot lysis and the concentration of thrombolytic used. Susceptibility to clot lysis is influenced by clot retraction, a process where the fibrin mesh within the thrombus contracts, resulting in a smaller, more mechanically-stable clot.[28] This releases entrapped, unbound plasminogen from within the thrombus and increases the concentration and formation of fibrin and the fibrin network.[29,30] In addition to clot retraction, lysis may also be affected by the ability of plasminogen activators to partially degrade fibrin and expose new plasminogen binding sites at concentrations that do not deplete plasminogen in plasma.[29] A study by Elnager, et al found that increasing the concentration of streptokinase applied to whole blood clots did not have much of an effect on clot weight, and it was proposed that the use of inappropriate doses of thrombolytic, and the limitations of natural fibrinolytic inductors such as plasminogen on lytic activity, negatively affected in vitro bioactivity.[31]

The study by Elnager, et al also demonstrates the importance of not relying solely on clot weight to determine lytic activity of plasminogen activators. While clot weight was not altered, a significant change in D-dimer concentration was detected that suggested a positive effect on clot lysis activity.[31] D-dimer, one of the final products of complete fibrinolysis, is a specific fibrin degradation product created from two adjacent fibrin monomers that indicates cross-linked fibrin-specific clot degradation by plasmin.[32-34] Of the coagulation assays available for diagnosis and prognosis of thromboembolic events such as deep vein thrombosis (DVT), PE, or infarction, the D-dimer is the most widely used.[34] Thrombolytic therapy, such as streptokinase and tenecteplase administration

during AMI, results in elevated serum levels of fibrinogen and fibrin degradation products, including D-dimer.[35] Studies by Melzer, et al and Brügger-Andersen, et al showed that tenecteplase administration resulted in markedly increased D-dimer concentrations in patients with massive and sub-massive PE and ST-segment elevation infarct (STEMI).[33,36] Based on previous studies, an increase in D-dimer concentration would suggest that tenecteplase has elicited its thrombolytic effects.

Analogous with findings from Elnager, et al,[31] the results of the D-dimer assay in the current study suggested more substantial lytic activity where the in vitro bioactivity assay did not. Based on clot weight degradation, it would be expected that the control samples would produce the greatest amount of D-dimer, while clots treated with tenecteplase would yield lower levels. This was not the case, however, as the control samples produced the least amount of D-dimer and clots treated with tenecteplase stored at 4.0°C produced more D-dimer (albeit not significantly) than any other temperature treatment. These findings provide further evidence that D-dimer is a more suitable indicator of fibrinolytic activity than clot weight. Although the amount of D-dimer detected in clot samples treated with tenecteplase stored at 35.5°C and 44.9°C had increased compared to pre-treatment levels, it was not as pronounced as refrigerated tenecteplase (however, this increase was also not significant). However, the exact extent to which the temperature treatments differed is difficult to determine as the maximum quantifiable D-dimer amount (35.2mg/L FEU for the Siemens CA-560 following automatic sample dilution)[37] was exceeded by several solutions, including all those stored at 4.0°C. In these instances, a value of 35mg/L was used. Despite this, the findings suggest that tenecteplase efficacy is affected by prolonged storage at high temperatures, potentially resulting in reduced clinical efficacy.

While studies have found that some drugs can tolerate extended periods of high temperature when stored in ESVs, it is evident that this is dependent on the drug and its chemical structure: the associated processes that occur during degradation (eg, elimination, rearrangement, or hydrolysis); reactions between the active compound and excipients; or interactions between the drug and storage container.[38-42] Therefore, while it may be perceived to be best practice, emergency service personnel should be discouraged from depleting older product before switching to newer stock as it is likely that some degradation may have occurred from numerous refrigeration-heat cycles. Alternatively, greater monitoring of drugs undergoing repeated refrigeration/heat cycling could be employed to help prevent unnecessary drug wastage. To alleviate temperature problems associated with ESV drug storage, implementation of a stock rotation scheme could be viable in some locations.[43] Where this is not effective, ambulances or critical care vehicles may be housed in climate-controlled garages or, depending on available funds,

mechanical medication cooling systems could be utilized.[44,45] Passive cooling systems could also be considered; however, they may reduce the efficiency of paramedics due to additional/heavier bag requirements.[45] Individual color-changing time-temperature indicator labels could also be applied to each medication, allowing users to easily and accurately monitor temperature exposure.[43,46] Suggestions such as these should be considered for tenecteplase storage to ensure it retains full stability and efficacy.

Limitations

In this investigation, tenecteplase was subjected to temperatures that exceeded those suggested by manufacturers for one eight-hour session. In the life cycle of a pharmaceutical carried by clinicians, emergency responders, and paramedics, this is not a long period of time. It is believed that had the study been conducted over a lengthier period with further cycles of refrigeration and warming, drug instability would have escalated in agreement with clinical observations from high temperature environments. This is speculation, however, and further study is warranted.

Additionally, the clots used in the current study would be considered extreme in a clinical scenario where the fibrinolytic system would naturally decrease thrombus severity. If an in vivo model was used, the tenecteplase samples in this study may have demonstrated further efficacy or a more discreet graded efficacy profile linked to temperature degradation. However, there are ethical considerations of delivering potentially compromised tenecteplase to a human thrombus. Rodent models of MI or thrombus were considered as a bioassay platform for determining thrombolytic activity of tenecteplase; however, these animal strains show physiologically faster clotting processes that are not directly applicable to human blood.[47] As such, the model used for this study was deemed the best approximation that could be utilized.

Finally, as stated previously, the maximum amount of D-dimer that could be quantified by the coagulation analyzer used was exceeded by several solutions in this study. The large amount of D-dimer produced is most likely due to the high concentration of tenecteplase added to the clots; if this was reduced to levels that were more clinically accurate, D-dimer levels may not have been as excessive. Alternatively, if there were more serum samples available, they could have been diluted further to extend the reportable range of the analyzer.

Conclusions

Based on the results of the current study, it is recommended that: storage of lyophilized tenecteplase should follow what is suggested by manufacturers (ie, below 30.0°C); storage temperatures should not continuously exceed 30.0°C for periods longer than eight hours; and lyophilized tenecteplase should not be subjected to refrigeration-heat cycling. It is also suggested that quantifying fibrin degradation products (eg, D-dimer) using an immunoassay should be the preferred technique for measuring clot lysis capacity over monitoring variations in clot weight, as this investigation has shown it to be a more accurate and reliable indicator of possible clinical effects.

Ultimately, the use of tenecteplase and other therapeutic proteins is an important aspect of emergency clinical medicine, particularly in rural and remote scenarios, that should be maintained and encouraged. However, the belief that it is best practice to use up old stock first must be deterred, as even in the lyophilized form, breakdown of the chemical integrity of protein drugs can occur very quickly in less-than-ideal conditions. In locations where prehospital treatment makes a vast contribution to the outcome of thrombus patients, control over the reconstitution and environmental preservation of medications like tenecteplase is critical in maintaining adequate pharmacological efficacy.

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Tables

Table 1. Biochemical Properties of Lyophilized Tenecteplase Stored for Eight Hours at 4.0°C, 35.5°C, or 44.9°C (n = 2)

Temperature Treatment	**Solution pH**		**Protein Concentration (mg/mL; 1:50 dilution)**	
	Mean (SD)	95% CI	Mean (SD)	95% CI
4°C	7.28 (SD = 0.007)	7.27-7.28	0.12 (SD = 0.015)	0.095-0.14
35.5°C	7.28 (SD = 0.000)	-	0.13 (SD = 0.004)	0.12-0.13
44.9°C	7.29 (SD = 0.000)	-	0.095 (SD = 0.000)	-

Please note: tenecteplase was reconstituted with water for injection to a nominal concentration of 5mg/mL prior to pH and concentration determination. Additionally, there were no significant differences between values.

Figures

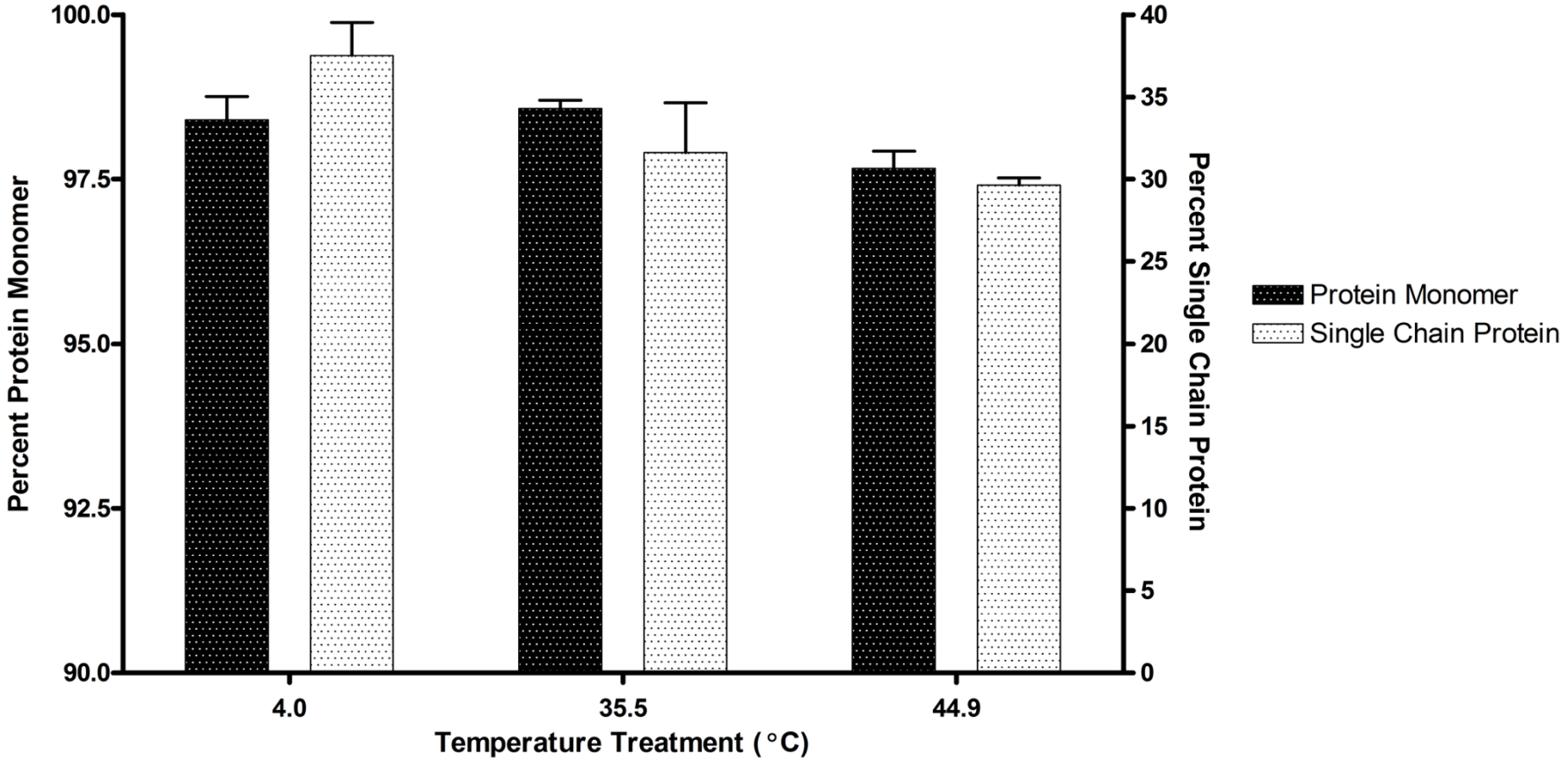

Figure 1. Percent protein monomer and single-chain protein remaining in tenecteplase samples subjected to temperatures of 4.0°C, 35.5°C, and 44.9°C for a period of eight hours. Data expressed as mean (SD), n = 2. Please note that there were no significant differences between values.

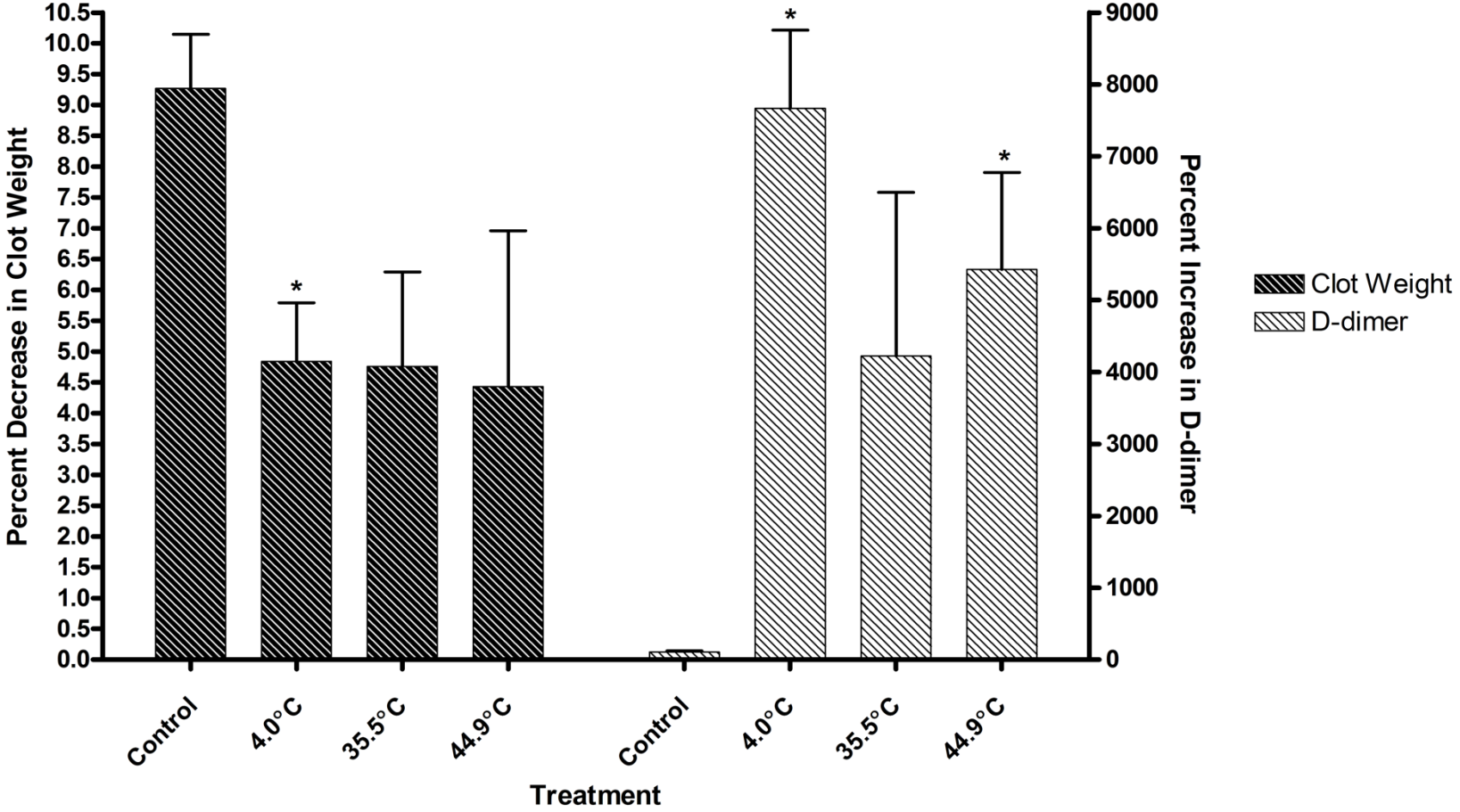

Figure 2. Differences in weight and detected fibrin degradation product (D-dimer) of human blood clots before versus after incubation with water (control) or solutions of tenecteplase subjected to temperatures of 4.0°C, 35.5°C, and 44.9°C for a period of eight hours. Data expressed as mean (SD), n = 2. *P <.04 versus control.

4. STUDY C – THE STABILITY OF EBC-46

4.1. Chapter summary

This study examines the effect of storage conditions on the stability of EBC-46, and how changes to these conditions affect its degradation. EBC-46 differs to the drugs studied previously as it is a natural compound still under clinical investigation, meaning stability studies have not yet been extensively performed. To conduct a comprehensive investigation, this study combines and adds to elements introduced in Chapters 2 and 3 by exploring a range of environmental conditions (specifically temperature, light and air exposure, and pH), and employing both concentration determinants of stability and *in vitro* analysis to define drug efficacy. This provides a more complete indication of how different storage conditions affect the stability of EBC-46 and uncovers the most appropriate storage environment to ensure drug potency, and consequently patient safety, is preserved.

EBC-46, also known as tigilanol tiglate, is a natural compound isolated from the kernels of a small tree found in North Queensland that possesses anticancer and wound-healing properties. Its mechanism of action is activation of PKC isoenzymes that regulate cellular functions such as proliferation and vasculature formation, resulting in tumour wall swelling and vascular damage within tumour sites that leads to haemorrhagic necrosis. It has showed promising results when trialled in domestic animals and is currently awaiting Phase IIA human efficacy trials. Under development by Australian life sciences company QBiotics Group, much of the research on EBC-46 efficacy is taking place at the QIMR Berghofer Medical Research Institute in Brisbane.

As EBC-46 moves through clinical trials, understanding how different environmental conditions can impact on its stability, particularly during storage, is increasingly important. Since the compound is a relatively new discovery (found in 2005) and still under development, degradation studies have not been extensively conducted. As well as being a necessity for new pharmaceuticals, this information could also highlight formulation requirements to prevent degradation of the active ingredient. As such, researchers from QIMR were interested in performing preliminary stability research on the pure EBC-46 product before it progressed further in its development. To ascertain a comprehensive indication of the compound's durability under storage, several factors that are known to affect drug stability and catalyse a range of degradation reactions (e.g. oxidation, reduction, hydrolysis) were explored. These included temperature, light, oxygen, and pH, with time of exposure ranging from six hours to one month. Additional information relating

to the methods used for this study have been included in the Appendix of this thesis (section 7.3). Determining the longevity of the purified product under a variety of conditions allows the most appropriate storage environment to be established, and therefore ensures the purified product remains stable for as long as possible. In addition to the factors mentioned previously, the metabolism of EBC-46 when exposed to hepatic and renal enzymes in an *ex* vivo model was also investigated. This would indicate where EBC-46 metabolism likely takes place in a biological model and highlight possible degradation pathways and products that may occur as a result. The analytical technique of high-performance liquid chromatography coupled with mass spectrometry was employed for all studies to determine the concentration of EBC-46 and its known primary metabolites/degradation products of EBC-211 and EBC-158.

The findings of this study will provide researchers and stakeholders with important and previously unknown data on the stability of the EBC-46 pure product, including causes of its degradation and what conditions are required to prolong its stability under storage. This information will be of great interest as EBC-46 moves through clinical trials, as optimisation of the drug's storage stability will increase its suitability as a widely used anticancer treatment.

The following manuscript was produced from this investigation and will soon be submitted for publication.

The influence of temperature, pH, air and light exposure, and cellular enzymatic degradation on the novel antitumor compound EBC-46

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Abstract

Purpose: EBC-46 is a naturally occurring, novel diterpene ester and protein kinase C activator that has shown promise as a treatment for a range of tumour types. As it is still early in its development, important stability and metabolic studies have not yet been conducted. As such, the aim of this investigation was to determine the impact of temperature, light, air, and pH on EBC-46 stability when stored, and explore its metabolism by renal and hepatic enzymes. **Methods:** EBC-46 in solution was subjected to a range of conditions to determine the effect of temperature, light (natural sunlight), oxygen, pH, and hepatic and renal enzyme interaction on its stability. High performance liquid chromatography coupled to mass spectrometry (LC-MS/MS) was used to monitor EBC-46 concentration degradation and degradation product/metabolite production. **Results:** The production of two less-active by-products (EBC-211 and EBC-158) was mostly affected by solution pH, but temperature had more of an impact on EBC-46 concentration. Specifically, EBC-46 stability was negatively impacted by high temperature, deviations from a pH of around 5, and sunlight exposure. EBC-46 concentration was not affected by the presence of oxygen or storage at 4°C. Mixing EBC-46 and 0.9% sodium chloride solution impeded degradation product development, regardless of storage temperature. Meanwhile, EBC-46 was metabolised by liver tissue, but not to a product detected in this study. EBC-46 concentration remained relatively stable in kidney tissues, but EBC-211 was rapidly produced. **Conclusion:** To increase stability and prevent degradation product formation, EBC-46 should be stored under refrigeration in a solution buffered to a pH of 5. Furthermore, it appears EBC-46 degrades to the less-active metabolite EBC-211 in a human physiological environment.

Introduction

EBC-46 (tigilanol tiglate, 12-Tigloyl-13-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one; $C_{30}H_{42}O_{10}$; 562.65 g/mol) is a novel diterpene ester and protein kinase C (PKC) activating compound, particularly of the –β isoforms that are known to induce endothelial cell permeability (Nagpala, Malik, Vuong & Lum 1996; Boyle et al. 2014). Isolated from the kernels of the fruit of Blushwood (*Fontainea picrosperma*), a small dioecious tree indigenous to North Queensland's Atherton Tablelands, EBC-46 is under development by QBiotics Pty. Ltd. (Adams 2013; Boyle et al. 2014; Grant et al. 2017).

PKC isoenzymes regulate many cellular functions, including cellular proliferation and vasculature formation, and their altered expression can result in tumour growth and metastasis; as such, inhibition of PKC signalling was previously identified as a potential cancer treatment (Totoń et al. 2011; Mochly-Rosen, Das & Grimes 2012). However, challenges with potential PKC-inhibiting drugs throughout their development, such as low patient tolerability and inadequate efficacy during clinical trials, caused focus to move away from these compounds (Mochly-Rosen, Das & Grimes 2012). Instead, more recent research on the tumour-supressing nature of PKC has brought PKC-activating drugs to the forefront (Isakov 2018). Efficacy studies have shown promising results, particularly the swelling and disruption of tumour-specific vessel morphology (Nufer, Yamada & Prow 2016). For example, treatment with the prototypic PKC-activating compound phorbol 12-myristate 13-acetate (PMA) resulted in remission of chemotherapy-resistant myelocytic malignancies (Han et al. 1998). EBC-46 and PMA are structurally related, however, EBC-46 is less hydrophobic due to short ester side-chains and hydroxylation of the B ring (Figure 1), and they differ in their selectivity and potency towards specific PKC isoforms (Boyle et al. 2014). Furthermore, while EBC-46 has been shown to be threefold-less potent for inhibiting tumour cell growth *in vitro* compared to PMA, it has a more enduring and successful anti-tumour effect *in vivo*; this is speculated to be the result of EBC-46 activating not only PKC isoforms, but other molecules with anti-cancer properties (Boyle et al. 2014).

Investigations into the therapeutic use of EBC-46 range from the intralesional treatment of solid tumours (Boyle et al. 2014), the topical treatment of complicated acute or chronic non-healing wounds (Dally et al. 2017), and as a potential chemotherapeutic topical agent for the non-invasive, less-destructive (compared to traditional therapy) treatment of non-melanoma skin cancers (Nufer, Yamada & Prow 2016). Trials in previously untreatable dogs, cats and horses have shown promising results for EBC-46 in the local treatment of a range of tumour types, including mast cell tumours, melanomas, squamous cell carcinomas, adenocarcinomas, and soft tissue sarcomas. Destruction of the tumour has been found to

occur within days, with rapid healing of the site and no significant adverse effects when used at therapeutic doses (Campbell et al. 2014).

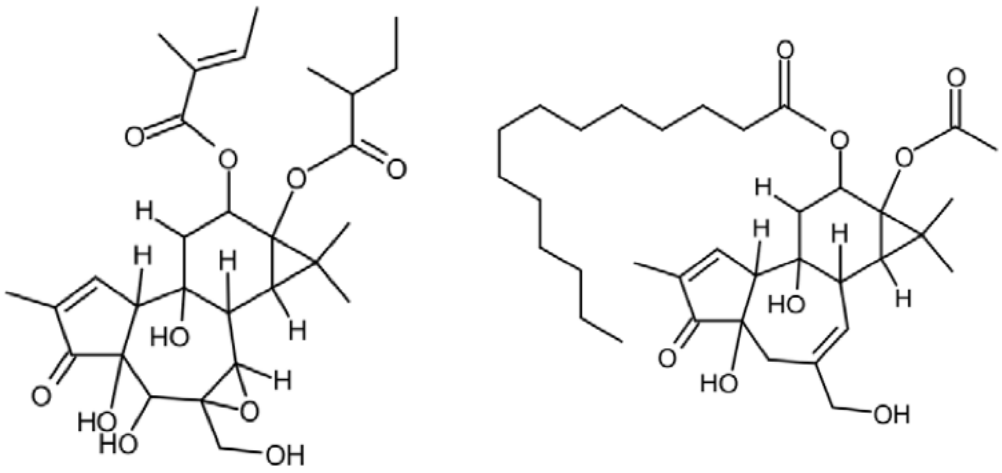

Fig. 1. Structure of EBC-46 (left) and PMA (right) (Boyle et al. 2014).

Stability testing is an essential component of the development stage of a new pharmaceutical. This involves exposing the drug to a range of environmental conditions in order to determine likely degradation pathways and products, and informs both formulation and storage requirements (Blessy, Patel, Prajapati & Agrawal 2014). Factors that are known to affect the stability of drugs in samples include temperature, light, oxidation, and enzymatic degradation (Briscoe & Hage 2009). Ensuring a stable purified sample is especially important for EBC-46; interest in the biostability of it and its derivatives grows as planning commences for a Phase IIA human efficacy clinical trial (QBiotics Group Limited 2018).

EBC-46 has been found to be relatively stable in both human whole blood (i.e. even in the presence of esterases) and phosphate-buffered solution over 24 hours (Henrys 2018); this suggests that the C12 and C13 esters may exhibit some steric hindrance, possibly due to the surrounding methyl groups (Figure 1). However, it is known from previous work by those at QIMR Berghofer Medical Research Institute that the formation of two predominant EBC-46 by-products is influenced by pH; in acidic environments, an increase in hydrogen ions results in hydrolysis of the epoxide in the B ring of EBC-46 to form EBC-158 ($C_{30}H_{44}O_{11}$; 580.66 g/mol), while at a pH greater than 5, elevated hydroxide ions induce Payne rearrangement of the epoxy group to form EBC-211 ($C_{30}H_{42}O_{10}$; 562.65 g/mol) (unpublished findings; Figure 2). As the modifications that produce EBC-158 inactivate EBC-46, it is believed to also be biologically inactive (Henrys 2018). EBC-211 differs in that it still elicits some wound-healing activity; however, this is reportedly reduced 100-fold compared to the parent compound (Moses 2016). Therefore, to ensure full efficacy of EBC-46, it is crucial that its stability is

retained during storage, and that its chemical breakdown into EBC-158 and EBC-211 is prevented.

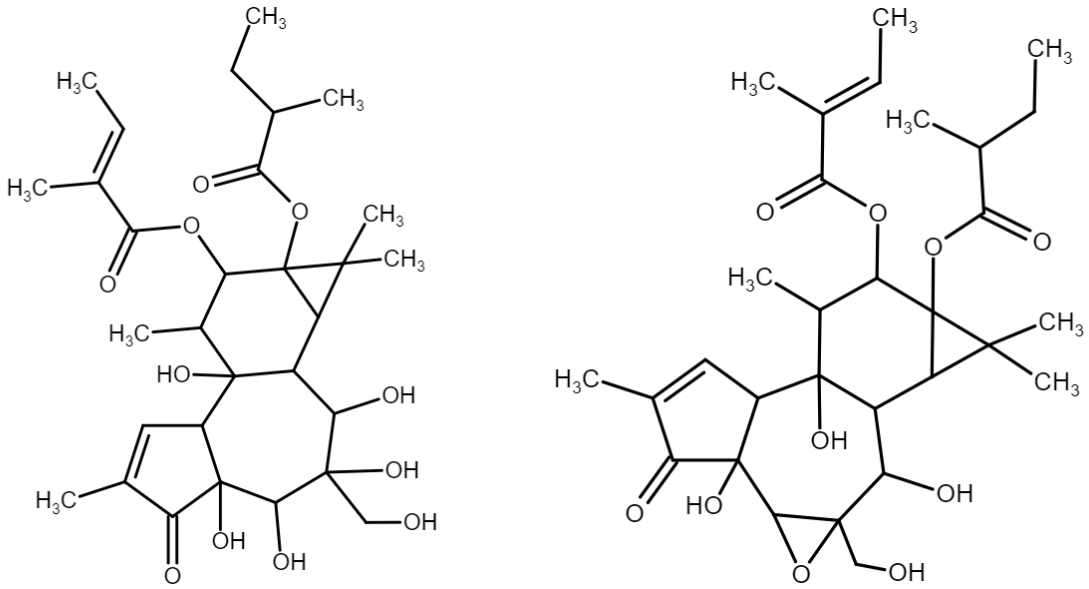

Fig. 2. Structure of EBC-158 (left) and EBC-211 (right).

As such, the aim of this study was to determine the effect of temperature, light (natural sunlight), air (oxygen) and pH on the stability of EBC-46 in storage over time, as well as investigating its metabolism by renal and hepatic enzymes *ex vivo*. The findings will provide valuable information on the proper formulation and storage conditions required to prolong stability of the purified product, and as a result, could increase the suitability of EBC-46 as an anti-cancer treatment.

Methods

Materials and reagents

All samples and reagents, including the master stock of EBC-46 used for the preparation of solutions (stored at 4°C prior to study commencement), were kindly supplied by QIMR Berghofer Medical Research Institute (Brisbane, QLD, Australia). Analysis was performed on a Shimazdu Prominence HPLC system (Shimadzu Scientific Instruments; Sydney, NSW, Australia) coupled to an Applied Biosystems/MDS SCIEX API3200 LC-MS/MS (Applied Biosystems Inc.; Foster City, CA, USA) run in the positive ion mode. A Halo RP-Amide column with dimensions of 4.6 x 150 mm, 2.7 µm (Advanced Materials Technology; Wilmington, DE, USA) was used for analysis with mobile phases consisting of 0.1% formic acid in water (mobile phase A) and in acetonitrile (mobile phase B). The analytical parameters and methods used for this study were developed and validated previously by researchers at QIMR Berghofer Medical Research Institute (findings unpublished).

The stability of EBC-46 over time when stored at 37°C

A solution of 2.2 mg/mL EBC-46 in ethanol was diluted in fetal bovine serum (FBS) to a final concentration of 0.1 mg/mL to ensure sufficient quantification under the analytical parameters chosen (as determined previously; findings unpublished). This was stored in a transparent plastic vial at 37°C for 144 hours, with duplicate aliquots removed and analysed by LC-MS/MS at 0, 24- and 144-hours following sample preparation. An EBC-46 standard (0.193 mg/mL), prepared previously, was analysed concurrently for concentration determination.

The effect of temperature, light and air on the stability of EBC-46

A solution containing 0.1 mg/mL EBC-46 in 10:90 v/v FBS and Hank's Balanced Salt Solution was prepared. Aliquots were removed, placed into transparent vials, and immediately subjected to one of five treatments: storage at 4°C for 96 hours; storage at room temperature (RT, approximately 25°C) for 96 hours; exposed to natural sunlight for six hours (in a clear glass vial); covered (to minimise light exposure) and gently bubbled with a continuous flow of oxygen on a laboratory rocker for 96 hours; and covered and gently bubbled with a continuous flow of nitrogen gas (to create an low-oxygen environment) on a laboratory rocker for 96 hours. Samples were immediately stored at 4°C following treatment, and were removed and warmed to RT prior to analysis by LC-MS/MS.

The effect of pH and temperature on the stability of EBC-46

Solutions containing 0.1 mg/mL EBC-46 and 10% FBS were adjusted to pH 3, 4, 5, 6, 7, 8 or 9 using appropriate buffers (0.01 M), while an additional solution was mixed with 0.9% sodium chloride solution. Aliquots were immediately removed from each solution for initial timepoint measurements and stored at 4°C until analysis. The remaining solutions were divided into four transparent plastic vials; two preparations were stored at 4°C and the other two at 37°C for a total of one month (28 days). During this time, aliquots were removed from each of the four preparations and immediately stored at 4°C until analysis, as follows: every two hours for a total of eight hours; every 24 hours for one week (seven days); at two weeks post-solution preparation (14 days); and finally, at one-month post-solution preparation (28 days). Prior to analysis by LC-MS/MS, aliquots were removed from 4°C and warmed to RT. Standards containing 0.1 mg/mL EBC-46, EBC-158 and EBC-211 were also analysed simultaneously, for concentration determination.

Metabolism of EBC-46 ex vivo

The following was based on a protocol used at QIMR Berghofer Medical Research Institute. Liver and kidney tissues from a six-month old male C57BL/6 mouse were mixed with 10x w/w 50 mM phosphate buffer, pH 7.5 and sonicated for 15 seconds on a Branson 250 sonifer (Branson Ultrasonics, Dansbury, CT, USA) at 40% duty cycle. After centrifugation at 2000 RPM for 10 min at 4°C the supernatant was removed, and an equal volume of buffer used to resuspend the pellets. Aliquots from each supernatant solution were also removed and stored at -20°C as a negative control. EBC-46 in ethanol was added to the resuspended pellet/supernatant solutions to a final concentration of 0.3 mg/mL prior to incubation at 37°C on a magnetic stirrer. Further aliquots were removed after three and 24 hours and stored at -20°C until analysis.

For analysis by LC-MS/MS, aliquots were thawed and vortexed with a 50:50 mixture of hexane and dichloromethane, followed by centrifugation for 10 minutes at 3000 RPM. Supernatant was removed and further solvent added to the tissue before additional centrifugation. All supernatant was then dried on a SpeedVac vacuum concentrator (Thermo Fisher Scientific, Scoresby, VIC, Australia) and redissolved in 100 µL of ethanol. To ensure adequate quantitation, solutions were further diluted (1:10) prior to analysis. Concentrations of EBC-46, EBC-211, and EBC-158 in pellet and supernatant samples were determined by LC-MS/MS. A standard curve, developed through analysis of EBC-46 solutions ranging from concentrations of 0.01 mg/mL to 0.20 mg/mL, was used to quantitate EBC-46 recovery.

Statistical analysis

ANOVA and Student's *t* test were used to determine statistical significance, with $P<0.05$ considered statistically significant (Prism version 4.02; GraphPad Software, San Diego, CA, USA).

Results

Simultaneous LC-MS/MS analysis of EBC-46, EBC-211 and EBC-158 was possible as there was adequate separation between each analyte; the retention times were 8.7 minutes, 8.2 minutes, and 6.7 – 6.9 minutes, respectively.

The stability of EBC-46 over time when stored at 37°C

EBC-46 degraded when subjected to 37°C, with 94.9% of the initial concentration remaining after 24 hours, and 66.0% remaining after 144 hours (Figure 3). Conversely, the concentration of the degradation product EBC-211 increased considerably following heat treatment. Compared to the initial concentration of 1.42 µg/mL, EBC-211 increased 4.47 µg/mL (315.4%) after 24 hours and 16.06 µg/mL (1134.2%) after 144 hours (Figure 3). This final amount was significantly greater than the previous timepoints ($P<0.0326$).

The effect of temperature, light and air on the stability of EBC-46

All samples exhibited EBC-46 degradation over the time period tested, while none contained a quantifiable amount of EBC-158.

Solutions subjected to nitrogen (oxygen-deficient) treatment showed the least amount of EBC-46 degradation overall, with 84.0% of the initial nominal concentration remaining after 96 hours; this resulted in significantly more EBC-46 remaining than all but the RT samples ($P<0.0435$). Nitrogen-treated solutions also contained significantly more EBC-211 than all other samples, with 2.0245 µg/mL present after 96 hours ($P<0.0011$) (Figure 4). Solutions subjected to air showed similar findings, containing 83.8% of the initial EBC-46 nominal concentration ($P<0.0464$) and 1.8185 µg/mL EBC-211 ($P<0.0017$) after 96 hours (Figure 4).

EBC-46 was also quite stable in solutions stored at 4°C, with 78.6% of the initial nominal concentration remaining after 96 hours. This was similar to solutions stored at RT that contained 76.7% of the initial nominal concentration at the same time point. However, solutions stored at 4°C produced significantly less ($P=0.0021$) EBC-211 than RT solutions, with 0.2351 µg/mL and 0.9839 µg/mL detected, respectively. Furthermore, 4°C solutions produced significantly less EBC-211 than all other treatments tested ($P<0.0058$) (Figure 4).

Of all solutions tested, those subjected to sunlight treatment showed the greatest degradation in EBC-46 overall, with 70.5% of the initial nominal concentration remaining after 6 hours. This amount was significantly less than all but the RT samples ($P<0.0495$). However, sunlight-treated solutions had the second lowest EBC-211 concentration of the treatments tested, containing 0.5463 µg/mL (Figure 4).

The effect of pH and temperature on the stability of EBC-46

For solutions stored at 4°C, those with a lower pH value (3 – 5) showed significantly greater EBC-46 stability than those with a mid- to high-range pH or mixed with NaCl ($P<0.0453$) after seven days, however, this observation had generally reversed after 28 days ($P<0.0464$) (Figure 5a). The production of EBC-211 at 4°C was minimal in solutions at low-range pH values (3 – 5), with significantly less EBC-211 present than in solutions with pH 6 and above ($P<0.0408$) that were also significantly different from each other ($P<0.0370$). Solutions at pH 8 (made with borate buffer) and pH 9 contained significantly more EBC-211 than all other solutions after 28 days ($P<0.0177$) (Figure 5b). While the production of EBC-158 was generally greater in solutions with low pH initially, after three days both pH 3 and 9 solutions contained significantly more EBC-158 than mid- to high-range pH (6 – 8) and NaCl solutions ($P<0.0482$). After 28 days, there were no significant observable differences in the EBC-158 concentration of any solution stored at 4°C (Figure 5c).

For solutions stored at 37°C, EBC-46 degraded similarly in all solutions until seven days had passed, after which those with a high pH (8 and 9) showed significantly more degradation than all other solutions up until the final time point ($P<0.0485$); pH 9 solutions were the least stable overall ($P<0.0037$) (Figure 6a). Additionally, solutions at high pH stored at 37°C developed significantly more EBC-211 than the other solutions over the time period analysed ($P<0.0109$), particularly pH 9, while solutions at low pH resisted EBC-211 production the longest and contained significantly less EBC-211 than the other solutions ($P<0.0423$) (Figure 6b). After one day, solutions at pH 9 stored at 37°C contained significantly more EBC-158 than any other solution ($P<0.0149$), however, after three and seven days, solutions at both pH 3 and 9 had developed significantly more EBC-158 than the other solutions ($P<0.0478$). After 14 days, only pH 3 solutions contained significantly more EBC-158 than any other solution, with this continuing through to the final time point ($P<0.0094$) (Figure 6c). Solutions mixed with NaCl resisted developing EBC-158 the longest, and at 14 days post-preparation had significantly less EBC-158 than all other solutions ($P<0.0384$).

Direct comparison between samples stored at 4°C and 37°C indicates that at a pH of 3 – 6 or when mixed with NaCl, EBC-46 was initially more stable at 37°C but soon degraded comparably to 4°C solutions for the remaining time period. This was similar for solutions at a higher pH, but by the final time point EBC-46 was significantly more stable in solutions stored at 4°C than in those stored at 37°C ($P<0.0130$). It was also found that at low pH, EBC-211 development in solutions stored at 37°C was initially significantly less than in 4°C solutions ($P<0.0244$), but both solutions behaved similarly for the remaining time period. Solutions at pH 5 and above and those mixed with NaCl behaved similarly, as those stored at 4°C initially

contained significantly more EBC-211 than 37°C solutions ($P<0.0280$). This quickly reversed though, with EBC-211 developing significantly in the warmer samples ($P<0.0309$). Solutions at low pH (3 and 4) that were stored at 37°C showed a steady development of EBC-158 and contained significantly greater amounts than solutions stored at 4°C from seven days onwards ($P<0.0019$). In mid-range pH solutions (5 – 8), EBC-158 production initially behaved similarly in both 4°C and 37°C solutions until 14 days had passed, where EBC-158 spiked in 37°C solutions. Despite this, these solutions contained less EBC-158 at the final time point than solutions stored at 4°C. At pH 9, EBC-158 developed significantly faster in 37°C solutions ($P<0.0498$), peaking after seven days before decreasing for the remaining time period. Solutions mixed with NaCl stored at 4°C and 37°C developed EBC-158 similarly.

Metabolism of EBC-46 ex vivo

EBC-46 behaved similarly in both liver samples; at the three-hour timepoint, these solutions contained significantly higher concentrations of EBC-46 than kidney samples ($P<0.0042$), but after 24 hours this difference was no longer significant. Specifically, EBC-46 in liver supernatant solutions decreased 45.3% between the 3 – 24 hours following preparation, while in liver pellet solutions this decrease was 38.5%. Conversely, EBC-46 concentration remained steady in both kidney supernatant and pellet solutions (Figure 7a).

While EBC-211 concentration was significantly greater in liver solutions after three hours ($P<0.0062$), kidney solutions exhibited a greater increase between the 3 – 24 hours following sample preparation. In liver supernatant and pellet solutions, EBC-211 concentration increased 34.0% and 40.7%, respectively. In kidney supernatant samples, EBC-211 concentration increased 110.1%, while kidney pellet samples showed an increase of 197.6%, the greatest of all tissues tested (Figure 7b).

The amount of EBC-158 in all samples was too low to accurately quantitate.

Discussion

The aim of these experiments was to determine the stability of EBC-46 when exposed to several stressors, such as temperature and pH changes, as well as certain metabolic products following incubation with isolates from kidney and liver tissue in an *ex vivo* model. There are several by-products formed during EBC-46 degradation, with two of the major ones that exhibit some activity being EBC-211 and EBC-158 (J Johns 2016, pers. comm., 26 July). pH in particular is believed to impact the formation of these degradation products; an acidic environment (pH value less than 5) results in EBC-158 formation, while a more alkaline environment (pH greater than 5) produces EBC-211 (so is therefore found *in vivo*). Due to this, a pH range of 4 – 5 is typically used for EBC-46 long-term storage and clinical work (J Johns 2016, pers. comm., 26 July). When discussing degradation by-product formation in the current investigation, it is important to note that their ratio to the parent compound is small; EBC-46 can be measured in mg/mL, while EBC-211 and EBC-158 are measured in µg/mL.

Temperature is one major factor of drug instability that was explored in this investigation, specifically storage at 4°C, RT (approximately 25°C), and 37°C. The impact of storing EBC-46 at 37°C (physiological temperature) for a total of 144 hours not only reveals that heat affects EBC-46 stability but could also give insight into possible degradation pathways within an *in vivo* human model. While time of exposure differed between heat treatments (144 hours for 37°C solutions, 96 hours for 4°C and RT solutions) as they constituted two separate studies, the initial EBC-46 concentration was consistent. This allows trends in the degradation of EBC-46 and the formation of by-products to be compared at a range of temperatures. The results showed that, compared to storage at 4°C and RT, storing EBC-46 at 37°C caused significant degradation while also resulting in a large increase in EBC-211; these findings are to be expected as there is a positive correlation between temperature and the rate at which reactions occur due to increased collisions between molecules (as described by the Arrhenius equation), including those resulting in degradation. When comparing storage at 4°C to RT, there was only marginally less EBC-46 degradation seen in 4°C solutions, yet they contained significantly less EBC-211; this could indicate that EBC-46 had degraded to a product not detected during analysis. Generally, the findings from the temperature studies were as expected: that heat negatively affects the rate at which EBC-46 degrades and results in the increased production of by-products.

Other contributing factors to drug degradation that were explored were photolysis and oxidation. Drugs exposed to solar, UV, and visible light can undergo photodegradation through free radical formation or intermolecular energy transfer as a result of light absorption and excitation (Ahmad et al. 2016). Thermal, free-radical, and nonfree-radical processes can

induce drug autoxidation, or the uncatalyzed oxidation of a substrate by molecular oxygen (Schmidt & Hecker 1975; Hovorka & Schöneich 2001). Phorbol esters are highly susceptible to autoxidation (Schmidt & Hecker 1975), and other naturally occurring, PKC-activating tigliane esters have been found to be sensitive to photodegradation; for example, the concentration of 12-deoxy-13,16-dioxy phorbol esters present in the seed oil of the *Jatropha curcas* Linneaus plant rapidly decrease under natural sunlight irradiation (Yunping et al. 2012; Kongmany et al. 2017). In the current study, subjection to natural sunlight for six hours resulted in the greatest EBC-46 degradation of all treatments tested, despite the exposure time being the least. However, EBC-46 in these solutions did not follow the degradation paths seen with other treatments, as they contained the second-lowest amount of EBC-211. Furthermore, LC-MS/MS detected a degradation product in these samples that eluted after EBC-46; this was a significant finding as it was not previously known how to obtain this metabolite. As it was not detected in solutions subjected to high temperature, it is assumed that light induced the formation of this degradation product. As elucidation of photochemical reaction mechanisms require a thorough knowledge of the drug and its physical characteristics (Ahmad et al. 2016), it is currently difficult to determine the exact identity of this by-product; however, PMA (the PKC-activating compound that is structurally related to EBC-46) stored in diffuse daylight at 25°C has been shown to produce a number of autoxidation products, including epoxide and aldehyde variants (Schmidt & Hecker 1975). Therefore, EBC-46 may follow similar degradation paths as a result of autoxidation following light exposure; however, further elucidation studies of the unknown degradation product would need to be performed to confirm this. Additionally, conducting the experiment in an oxygen-free environment (achieved using inert gas and degassed solvents) would also confirm if the by-product is formed through light-initiated oxidation.

The oxygen tests, where a nitrogen gas purge was used to achieve a low-oxygen atmosphere, also produced interesting results; while these solutions contained more EBC-46 than any other treatment tested, they also had the highest concentration of EBC-211, particularly the nitrogen-treated solutions. Similar to the light-treated solutions, these findings suggest there may have been other products forming in the oxygen- and nitrogen-treated solutions that were interfering with either the EBC-46 or EBC-211 chromatogram peaks; again, further investigation is required to verify this. It should also be noted that the solvents used in the nitrogen-purged samples were not previously degassed, therefore, findings are based on an oxygen-deficient, and not oxygen-free, environment.

To gauge the effect of pH on EBC-46 degradation and by-product formation, a wide range of pH values were studied over a prolonged period of 28 days and were accompanied by temperature exposure. Including heat treatment simultaneously accelerates any potential

degradation while also confirming the stabilising effect of low temperature. Acetate, phosphate, and borate buffers were utilised to achieve a pH range of 3 – 9. Two solutions of pH 6 (one buffered with acetate, the other with phosphate) and pH 8 (one buffered with phosphate, the other with borate) were included to account for possible buffer effects. The degradation of EBC-46 mixed with 0.9% (or normal) saline solution was also explored as it is a commonly utilised intravenous fluid suitable for use in any future *in vivo* studies, and has a pH near 5 that is believed to be the most appropriate for minimal degradation by-product formation.

EBC-46 stored at 37°C degraded faster at alkaline pH, especially pH 9. As predicted, greater concentrations of EBC-211 were found in solutions with high pH, EBC-158 generally developed more in acidic solutions (particularly pH 3 and 4), and moderate levels of each were present in solutions at mid-pH. pH 8 solutions with borate buffer behaved unexpectedly in that they contained the second-highest amount of EBC-158 (usually produced under acidic conditions) of all solutions tested; conversely, pH 8 solutions with phosphate buffer behaved as predicted, with only low levels of EBC-158.

Solutions kept at 4°C behaved as expected, with acidic solutions containing less EBC-211 than alkaline solutions. Generally, EBC-158 showed no trends in its production; however, there were unexpected results with pH 9 solutions, where the concentration of EBC-158 was as high, or higher, than pH 3 solutions. However, any increase in EBC-158, regardless of solution pH, was relatively minimal. While these findings may suggest that EBC-46 kept at 4°C predominantly degraded to EBC-211, it should be noted that changes in EBC-46 concentration were only minor in context. Specifically, the concentration range at the initial timepoint was 0.081 mg/mL – 0.098 mg/mL, while at the final timepoint it was 0.088 mg/mL – 0.098 mg/mL. Additionally, in all solutions there was less EBC-211 present at the final timepoint than there was initially, and EBC-158 concentration only increased by a minimal amount. Considering this, EBC-46 is very stable when stored at 4°C regardless of solution pH; this is very likely a result of the rate of degradation being slowed by low temperature, and corroborates with other studies on similar compounds (Ahluwalia, Singh & Sharma 2018).

EBC-46 was also mixed with 0.9% saline solution, a commonly used intravenous fluid. As stated previously, normal saline is typically acidic with a pH of approximately 5.5 (Reddi 2013). EBC-46 stability and EBC-211 production in these solutions was comparable to those buffered to a similar pH, i.e. EBC-46 was stable and EBC-211 concentration remained low. However, saline solutions were found to contain lower levels of EBC-158 following 28 days of storage at 4°C than those of a similar pH, while those stored at 37°C resisted EBC-158 formation longer than any other solution tested. A possible explanation for this is that the increased ionic

strength caused a decrease in the rate of epoxide hydrolysis, as it has been demonstrated previously that the addition of chloride ions produces specific effects on this reaction (Whalen & Ross 1976). These findings indicate that using normal saline solution for EBC-46 storage may yield more benefits than buffered solutions of similar pH, not only in terms of decreased by-product formation but also increased suitability for possible future *in vivo* applications; however, further research is required.

Overall, temperature had a greater impact on EBC-46 stability than pH, with solutions stored at 4°C remaining more stable than those stored at 37°C. Also, while pH did influence the ratio at which EBC-211 and EBC-158 was produced, more of each by-product developed in solutions stored at 37°C than in those stored at 4°C. Previous studies have shown that heat treatment of seed meal containing naturally occurring tigliane diterpenes reduces their levels, allowing for safe animal consumption (Goel, Makkar, Francis & Becker 2007). Our study corroborates with these findings, as high temperatures were demonstrated to have a destabilising effect on EBC-46; again, this was to be expected due to the impact of temperature on reaction rates. Therefore, to maximise EBC-46 stability while avoiding excess production of either metabolite, a low temperature and mid-range solution pH (or 0.9% saline solution) should be considered for storage. This temperature profile may have implications for use *in vivo* when the anticipated systemic and cellular conditions will be at 37°C, contributing to accelerated parent compound degradation.

As mentioned previously, there were some discrepancies between solutions of the same pH that were made using different buffers. For pH 6 solutions made using acetate or phosphate buffer, inconsistencies were only significant for EBC-211, particularly in solutions stored at 4°C where acetate-buffered solutions contained higher concentrations. There were, however, more significant variances between pH 8 solutions buffered with phosphate or borate, especially for solutions stored at 37°C where the concentration of all forms of EBC differed significantly between buffers. The increased degradation of EBC-46 seen in borate-buffered solutions suggests that heat treatment may have had an impact on the integrity of the buffer; this could be confirmed by repeating the experiment using another buffer with a pH range of 8–9, such as 2-amino-2-methyl-1,3-propanediol (AMPD), and comparing the findings with both the phosphate and borate buffer solutions. In addition to this, other possible heat effects were observed. Five days following solution preparation, some samples had a cloudy appearance, particularly those at mid-range pH (5 – 7). While warming the samples stored at 4°C reduced this, those at pH 4 and 5 still had a translucent appearance. For solutions stored at 37°C, those with a pH of 4 – 7 (and, to a lesser extent, pH 8) were translucent; it is unknown whether cooling these samples to room temperature would have improved their appearance. Although

FBS was added to prevent EBC-46 from precipitating, it is suspected that this is what occurred in these solutions.

Another important aspect of a drug's suitability is its interaction with metabolic tissues and their associated enzymes, particularly within the liver and kidneys where metabolism and excretion primarily occur. For EBC-46 in particular, esterase activity could conceivably remove the C12 secondary ester (a product isolated from the plant as EBC-186), or the C12 and C13 esters (not yet seen in the plant). The C20 glucuronide could also be removed, as well as other reactions involving the 6,7-epoxy group or the two double bonds in the molecule (P Parsons 2016, pers. comm., 16 August). It is important to determine how EBC-46 is structurally modified following interaction with liver and kidney tissues so that the impact on clinical efficacy can be investigated. As such, the aim of this study was to elucidate how EBC-46 may be chemically altered *in vivo*. Utilising both pellet (tissue) and supernatant (fluid) provides an overall picture of EBC-46 metabolism as enzyme activity is shared between them (Gacko et al. 2004).

It was found that incubating EBC-46 with kidney tissues resulted in a high concentration of EBC-211. This suggests that, if prior structural changes did not occur *in vivo* and EBC-46 were to reach the kidneys intact, it would be primarily excreted as the less-active metabolite EBC-211. However, other findings indicate this is unlikely; EBC-46 concentration decreased substantially in liver solutions, demonstrating it would potentially undergo hepatic metabolism prior to reaching the kidneys. Interestingly, while EBC-46 degraded substantially and EBC-211 concentration remained steady in the liver solutions, EBC-158 was not detected. This suggests that the liver may predominately metabolise EBC-46 to a by-product not detected under the analytical conditions used in this study thus completely inactivating the drug. This is highly likely as epoxide hydrolysis (via epoxide hydrolases) is only one potential metabolic process (Kodani & Hammock 2015); others include hydroxylation and glucuronidation. For instance, liver carboxylesterases, acylcarnitine hydrolase, and diacylglycerol lipase have all been found to detoxify or hydrolyse PMA, while another study has shown that this compound is also metabolised by β-glucuronidase to form a glucuronic acid conjugate (O'Brien, Saladik, Sina & Mullin 1982; Goel, Makkar, Francis & Becker 2007). There are also circumstances during Payne rearrangement (the process required to form EBC-211) where *in situ* opening of the species can occur, forming an epoxy alcohol moiety (Hanson 2004). Therefore, further investigation using different analytical conditions is required so that other possible metabolic products may be elucidated.

Generally, EBC-46 behaved similarly in pellet and supernatant samples from the same tissue, while EBC-211 developed more quickly in pellet samples. As the formation of EBC-211

through Payne rearrangement is greatly influenced by pH (Hanson 2004), these findings indicate that the liver and kidney tissues provided a more alkaline environment than the supernatant, possibly as a result of natural buffering systems (Hopkins & Sharma 2019). However, as EBC-46 is a novel drug that is still early in its development, and the sample size was only small for this study, it is difficult to draw any further conclusions about the reason for the EBC-211 concentration discrepancies. Adding to this, while EBC-158 was not quantifiable under the specific analytical conditions used, it cannot be conclusively established that it was not present in trace amounts. The existence of EBC-158 would indicate quite different metabolic conditions than those required for EBC-211 production. Again, further investigation of EBC-46 metabolism by hepatic and renal homogenates (with increased sample numbers) is warranted.

As indicated previously, a major limitation of this study was low sample numbers due to the restricted availability of the EBC-46 pure product. Furthermore, the master stock of EBC-46 pure product used to prepare all solutions in this study had been made and stored at 4°C for a time prior to study commencement, and a degree of degradation may have already occurred by the time the solutions were prepared. As a consequence of these limitations, any conclusions drawn from the results cannot be considered definitive without further investigation. However, the preliminary findings do suggest that hepatic tissues metabolise EBC-46 to a greater extent than renal tissues and that, in storage, the stability of EBC-46 is dependent on temperature and light exposure, possibly as a consequence of autoxidation. Furthermore, storing EBC-46 at low temperature increased its stability (when compared to high temperature) by increasing its resistance to pH degradation, and when stored at high temperature, EBC-46 was less stable at high pH (greater than around pH 6). The effect of pH on EBC-46 metabolite production coincided with findings from previous research, in that more EBC-211 was produced at high pH (greater than around pH 5) and more EBC-158 was produced at low pH. However, sunlight exposure resulted in a different metabolite whose method of production was previously unknown. Interestingly, temperature had little effect on solutions containing normal saline; even at high temperature, EBC-46 remained relatively stable and formation of metabolites was minimal. Overall though, solutions stored at low temperature with a mid-range pH showed the greatest EBC-46 stability and produced the least amount of either metabolite. Therefore, the findings of this investigation suggest that the EBC-46 pure product should be stored under refrigeration in a solution buffered to a pH of 5. These conditions appear to be optimal for ensuring the anticancer and wound-healing properties of this promising compound are retained under storage. Furthermore, within a human physiological environment that strictly maintains a pH of 7.40 (Hopkins & Sharma 2019), it is expected that EBC-46 will be metabolised to EBC-211.

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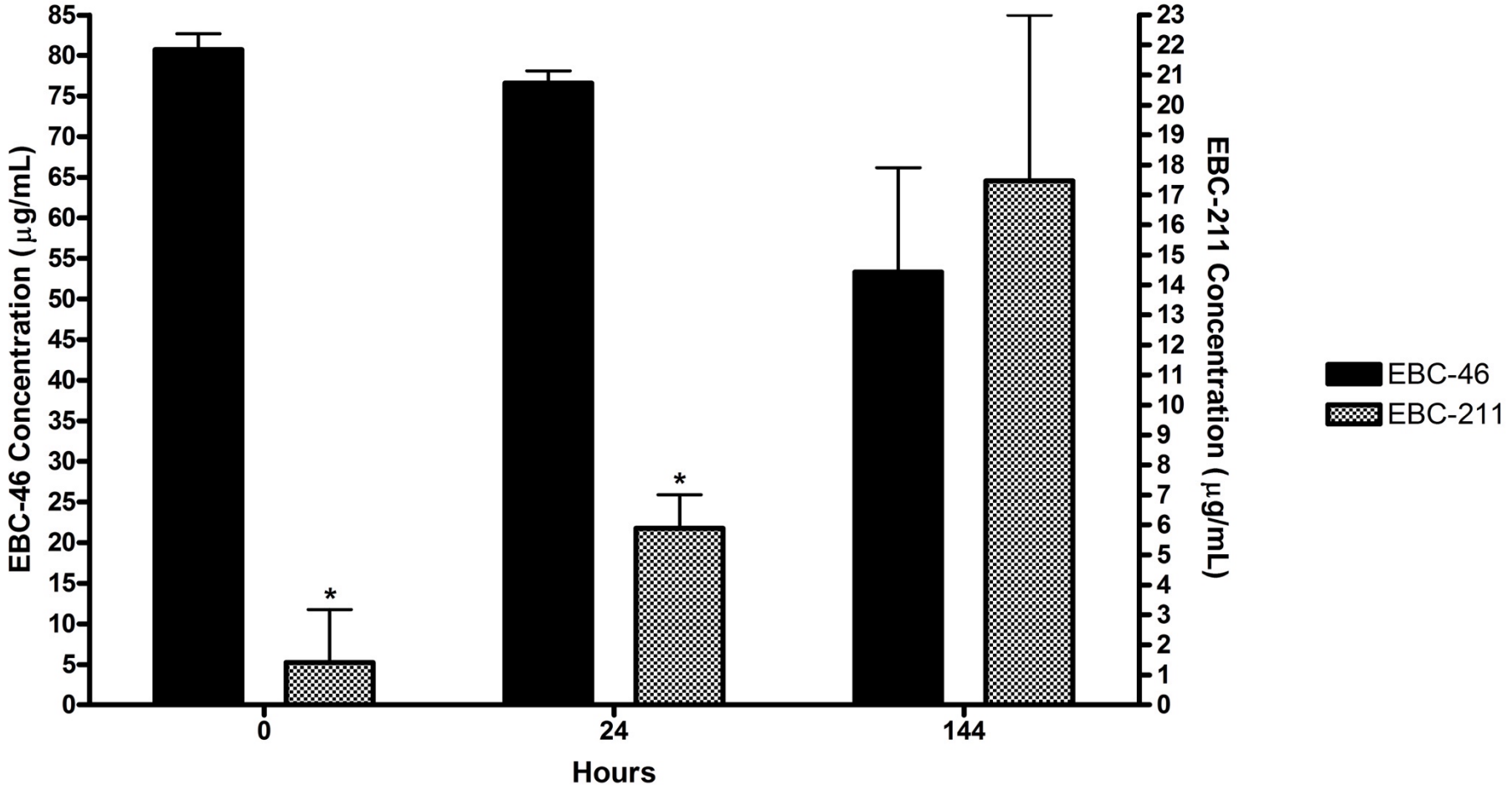

Fig. 3. Changes in EBC-46 and EBC-211 concentration over 144 hours when stored at 37°C. Data expressed as mean ± SD, n=2. *P<0.0326 vs. EBC-211 at 144 hours.

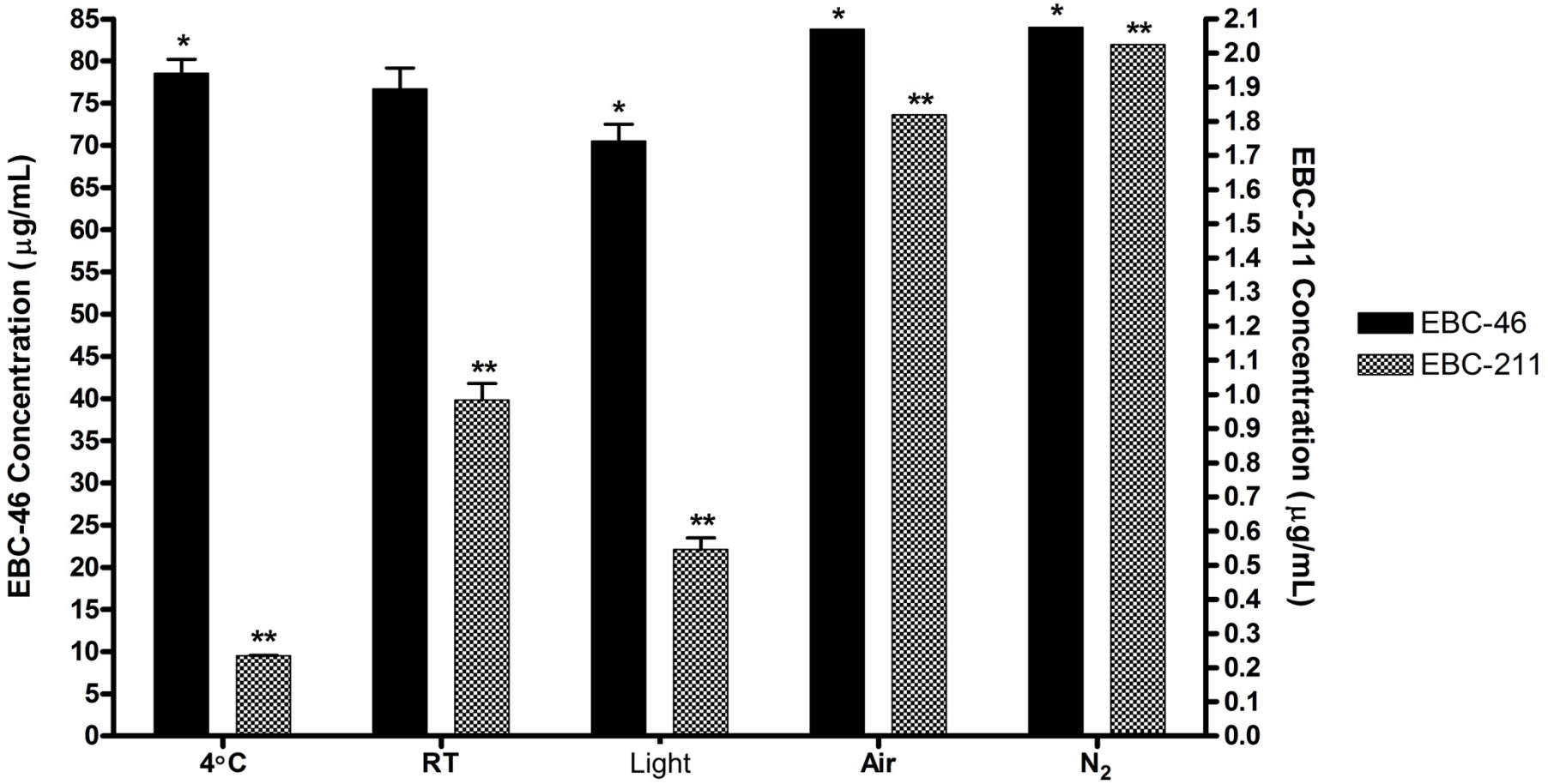

Fig. 4. Final solution concentration of EBC-46 and EBC-211 following exposure to 4°C (n=2) or room temperature (RT) (n=2) for 96 hours, natural sunlight (n=2) for six hours, and air (n=1) or nitrogen gas (N_2) (n=1) for 96 hours. Data expressed as mean ± SD. *P<0.0495 vs. all other EBC-46 concentrations except RT; **P<0.0091 vs. all other EBC-211 concentrations.

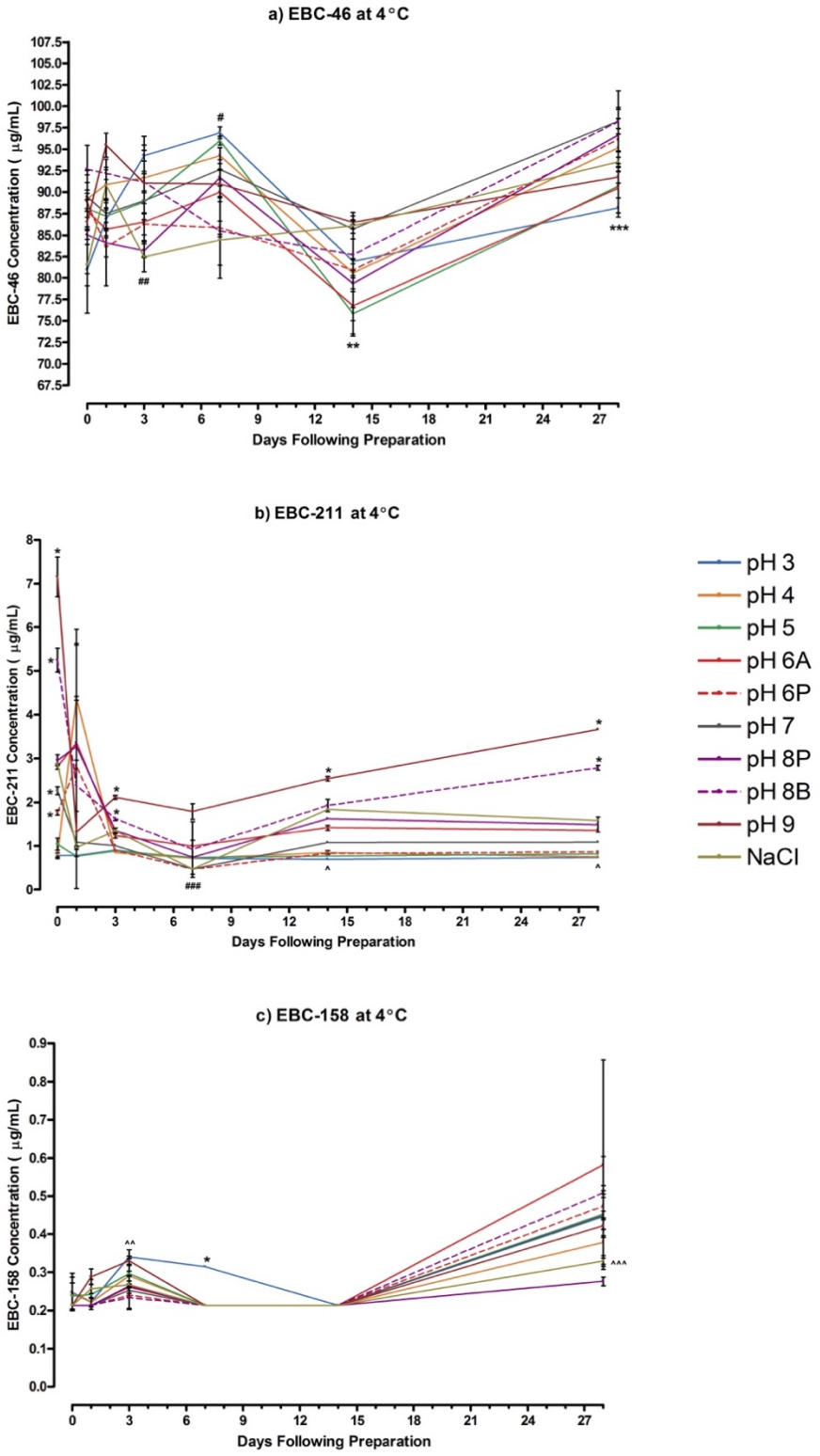

Fig. 5. Changes in solution concentration of a) EBC-46, b) EBC-211 and c) EBC-158 stored at 4°C over 28 days when buffered to a pH of 3, 4, 5, 6 (A = with acetate buffer, P = with phosphate buffer), 7, 8 (B = with borate buffer) or 9, or when mixed with 0.9% NaCl solution. Data expressed as mean ± SD, n=2; statistics are for each individual timepoint. *P<0.0362 vs. all other solutions; **P<0.0407 vs. pH 7, pH 9, NaCl; ***P<0.0318 vs. pH 8P, pH 8B, NaCl; #P<0.0312 vs. pH 6P, pH 8P; ##P<0.0290 vs. pH 3, pH 8B, pH 9; ###P<0.0097 vs. pH 3 – 6A, pH 9; ^P<0.0317 vs. pH 6A, pH 7 – NaCl; ^^P<0.0482 vs. pH 6A, pH 7, pH 8B, NaCl; ^^^P<0.0306 vs. pH 6P – 8P.

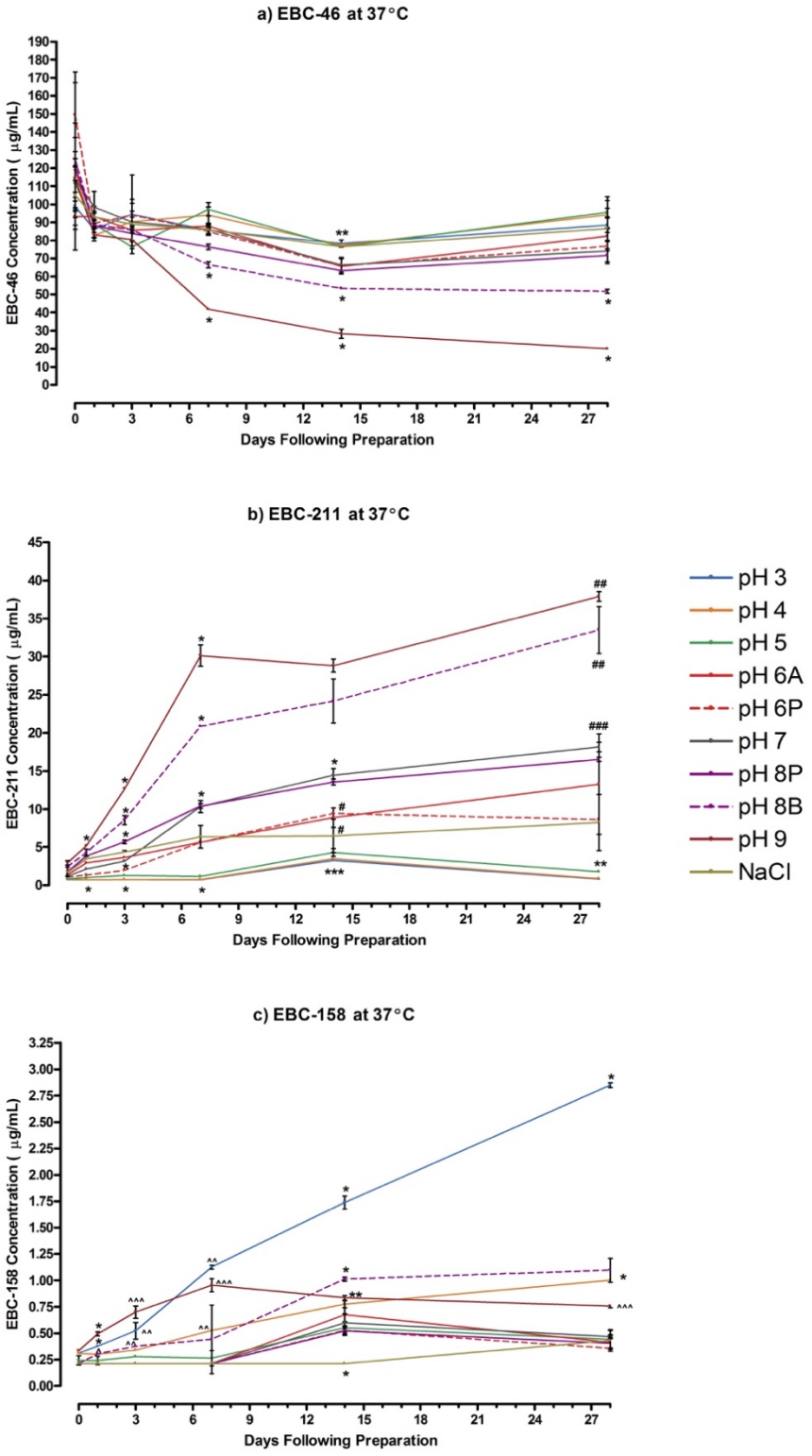

Fig. 6. Changes in solution concentration of a) EBC-46, b) EBC-211 and c) EBC-158 stored at 37°C over 28 days when buffered to a pH of 3, 4, 5, 6 (A = with acetate buffer, P = with phosphate buffer), 7, 8 (B = with borate buffer) or 9, or when mixed with 0.9% NaCl solution. Data expressed as mean ± SD, n=2; statistics are for each individual timepoint. *P<0.0492 vs. all other solutions; **P<0.0126 vs. pH 6P, pH 8P; ***P<0.0423 vs. pH 6A – 9; #P<0.0205 vs. pH 8B, pH 9; ##P<0.0203 vs. pH 3 – 5, pH 6P – 8P, NaCl; ###P<0.0022 vs. pH 3 – 5, pH 6P; ^P<0.0049 vs. pH 6A – 8P, NaCl; ^^P<0.0478 vs. pH 5 – 8P, NaCl; ^^^P<0.0468 vs. pH 4 – 8P, NaCl.

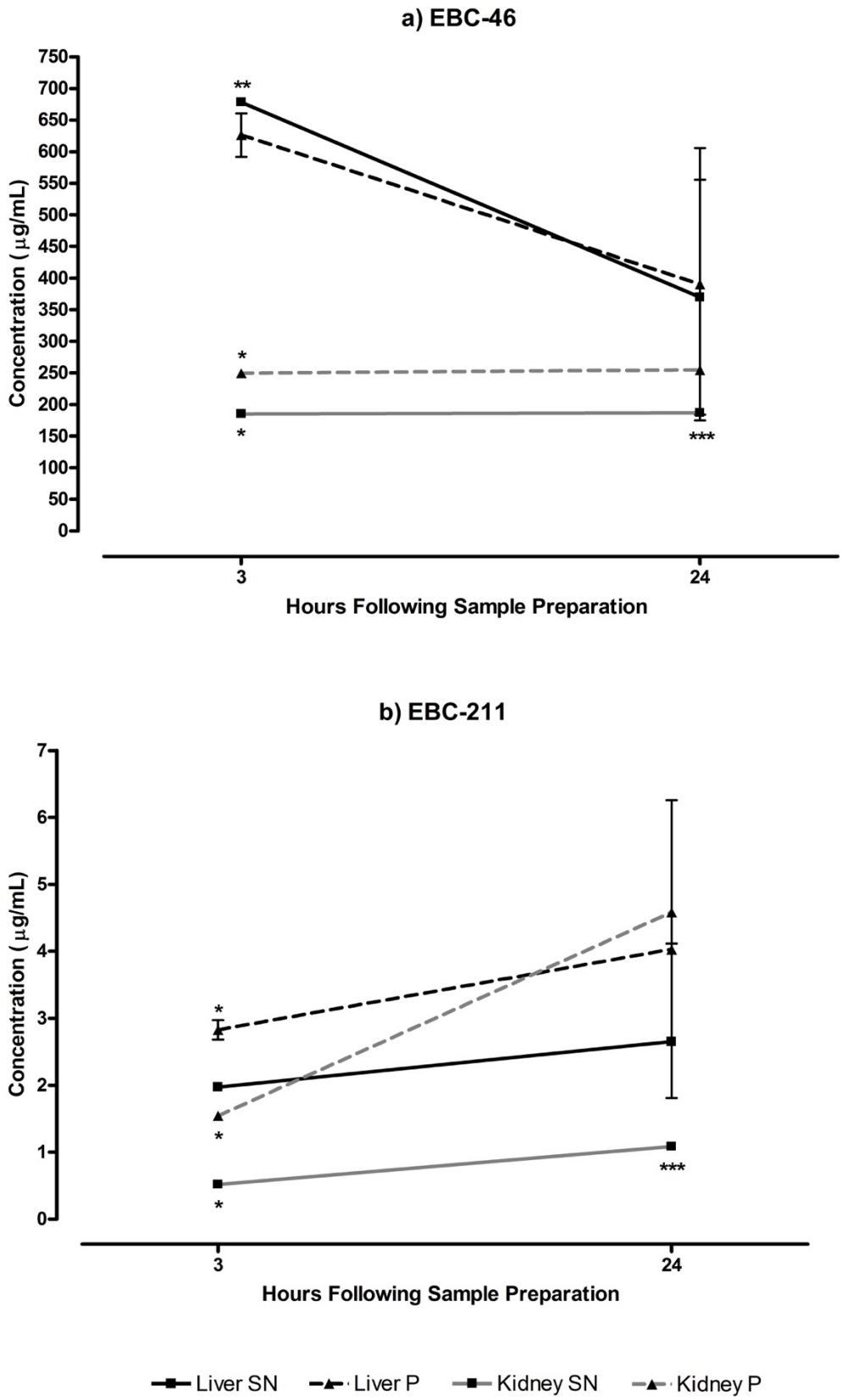

Fig. 7. Changes in concentration of a) EBC-46 and b) EBC-211 after incubation with liver (n=2) or kidney (n=1) supernatant (SN) and pellet (P) for 24 hours. Data expressed as mean ± SD; statistics are for each individual timepoint. *P<0.0143 vs. all other tissues; **P<0.0001 vs. all other tissues except liver pellet; ***P<0.0001 vs. kidney pellet.

5. THESIS SUMMARY

This investigation highlights the importance of understanding how storage conditions impact on the physical, chemical and biological stability of pharmaceuticals. There are many factors that can initiate degradation, including temperature, concentration, solution pH, diluent interactions, light exposure, and chemical processes such as oxidation and hydrolysis. If these are not properly controlled and the physical and/or chemical stability of a drug is negatively affected, there is a possibility that patient safety could be compromised; this can occur through decreased efficacy, diminished patient tolerability, and toxic metabolite production, among others (Kommanaboyina & Rhodes 1999). Adverse effects are particularly concerning for drugs used during surgery or to stabilise ICU patients (e.g. remifentanil and propofol), or for emergency medication that is required for life threatening conditions (e.g. tenecteplase). Furthermore, determining stability under various storage conditions is fundamental in ensuring novel compounds in the early stages of development (such as EBC-46) are suitable for eventual widespread use.

All studies in this investigation involved collaboration with external interested parties, formally with Rockhampton Hospital (Study A) and QIMR Berghofer Medical Research Institute (Study C), and informally with the Queensland Ambulance Service (Study B). As such, the information gathered has direct relevance and immediate applicability to clinicians and researchers in the medical field. Analytical techniques, predominantly high-performance liquid chromatography, were extensively employed throughout for drug concentration determination.

5.1. Summary of key findings

5.1.1. STUDY A – The stability of remifentanil and propofol in combination

The stability of remifentanil in isolation was not affected by initial concentration or when mixed with water or saline solution, where the pH remained less than 4. However, reconstituting remifentanil with alkaline sodium bicarbonate solution resulted in rapid, significant degradation. Furthermore, remifentanil degraded much faster when mixed with propofol, whose pH is approximately 7.7, than when in isolation. In addition to the presence of propofol, remifentanil degradation in these mixtures was affected by the initial remifentanil concentration (higher concentrations were more stable than low) and the diluent used/solution pH. In all instances where remifentanil instability was observed, it appeared to degrade to its primary metabolite, remifentanil acid. Conversely, propofol

remained stable in isolation and when mixed with remifentanil. Where a small amount of propofol degradation was detected, remifentanil concentration had more of an impact than solution pH, with higher concentrations resulting in decreased propofol stability.

Overall, a high solution pH increased remifentanil degradation, both in isolation and when mixed with propofol, and appeared to have the most influence on remifentanil stability. The most stable mixture, with over 90% of the initial remifentanil concentration present for up to four hours and 96.5% of the initial propofol concentration remaining after 24 hours, had the lowest pH (around 6.3) and contained 50 µg/mL of remifentanil reconstituted with 20% saline.

5.1.2. STUDY B – The stability of lyophilised tenecteplase at high temperatures

Exposing lyophilised tenecteplase to temperatures exceeding the suggested storage temperature of 30°C for one continuous eight-hour period negatively altered its stability and efficacy, and these effects were amplified as temperature increased; tenecteplase stored at 4°C was the least affected of the temperature treatments. Where temperature did impact on tenecteplase stability, the most affected aspects were structural conformation and *in vitro* efficacy. Following exposure to temperatures of 35.5°C and 44.9°C, the amount of protein monomer and single chain protein decreased; both are evidence of fragmentation of the tenecteplase molecule and can affect fibrin specificity. When these solutions were incubated with blood clots, the amount of fibrin degradation product (D-dimer) detected had also decreased, indicating reduced capacity for clot destruction. An additional discovery was the decreased solubility of the solid drug following heat treatment, with more care required during reconstitution to ensure a clear, uniform solution that was free of particulates. If this is not achieved, the concentration and efficacy of the solution would be reduced.

The findings of this study indicate that if tenecteplase is exposed to prolonged temperatures that exceed manufacturers recommendations, e.g. within a paramedic drug bag without adequate temperature control, its efficacy as a thrombolytic (and, consequently, the welfare of patients) could be compromised. It is therefore recommended that measures be put in place to ensure proper storage conditions are observed.

5.1.3. STUDY C – The stability of EBC-46

The stability of EBC-46 during storage was negatively impacted by heat, sunlight exposure, and high solution pH. Additionally, liver tissues were found to degrade EBC-46 to a further extent than kidney tissues in an *ex vivo* model. On the contrary, EBC-46

remained quite stable when stored at 4°C, room temperature, and at low pH, and was not affected by the presence/lack of oxygen or when incubated with kidney tissues. The formation of less-active metabolites was mostly affected by high temperature and solution pH, with higher pH producing more EBC-211 and lower pH producing more EBC-158. When mixed with 0.9% sodium chloride solution (pH of approximately 5), EBC-46 concentration remained stable and the production of either metabolite was minimised. Interestingly, solutions exposed to both oxygenated and deoxygenated environments produced a large amount of EBC-211, despite EBC-46 concentration remaining quite stable, while sunlight-treated solutions contained little EBC-211 and instead produced another metabolite altogether. Furthermore, while EBC-46 was more stable when incubated with kidney tissues than liver tissues, increases in EBC-211 were greater.

Overall, the findings indicate that, to maximise EBC-46 concentration while minimising metabolite production, it should be stored under refrigeration at a pH of 5 (or mixed with 0.9% sodium chloride solution) and protected from light. Additionally, it appears EBC-46 may undergo hepatic metabolism, however, this is not conclusive.

5.2. Clinical outcomes

5.2.1. STUDY A – The stability of remifentanil and propofol in combination

This study provides further knowledge on the impact of pH and diluent on remifentanil and propofol stability, and how these affect the viability of using the drugs as a mixture. Firstly, the demonstrated stability of the drugs in isolation, particularly reconstituted remifentanil, suggests that clinicians working in busy environments could have the drug prepared for use for up to 24 hours. This would increase the efficiency of patient care, and conceivably decrease the occurrence of dosing errors. In terms of using the drugs in combination, our preliminary findings indicate that if the solution contained a remifentanil concentration of 50 µg/mL, was appropriately buffered to a pH of 6, and was stored in glass, a remifentanil-propofol mixture may be feasible when used for no more than four hours. This could be of great interest to clinicians working with patients who require a variety of infusions, as only one IV line is required for both the analgesic and anaesthetic. Administering a mixture of the drugs also provides the advantage of immediate initiation and termination of the infusion when needed, and as both remifentanil and propofol have a rapid clearance rate, the patient will recover quickly; this is of particular benefit for outpatient surgery. A flow-on effect of faster recovery is earlier discharge, especially if patients can bypass the recovery room, and this would decrease costs for clinics and patients alike. Finally, this study may

assist clinicians in identifying areas where further efficiencies could be made to improve patient compliance and comfort, such as the co-infusion of other analgesic/anaesthetic combinations.

5.2.2. STUDY B – The stability of lyophilised tenecteplase at high temperatures

This study demonstrates how exposure to temperatures that have been shown to occur during summer in areas such as Central Queensland can impact on the efficacy of the drug tenecteplase. The results highlight the importance of ensuring the recommended storage temperature is adhered to and suggest that improvements made in this area are likely to result in better patient outcomes. Therefore, more stringent regulations over the way tenecteplase and drugs like it are stored and transported in emergency vehicles may be enforced. For example, a stock rotation scheme could be introduced to ensure drugs are not continuously exposed to high temperatures, although this is subject to how often the vehicle can return to its base and may not be possible in some remote areas. Another potential outcome is the application of affordable, easy-to-understand time-temperature indicators to the boxes of vulnerable pharmaceuticals. These labels have a measurable characteristic (e.g. colour) that irreversibly and continuously changes over time in response to temperature exposure, allowing rapid identification of any drugs that may be affected (Manjunath 2018). Additionally, small cooling systems could be placed in ambulances that would remove the need for regular stock rotation or time-temperature indicators, although this has the limitations of being costly and requiring adequate space. Regardless of the strategy that may be implemented, Queensland Ambulance Service and similar organisations will, at the very least, be more aware of the importance of adhering to recommended storage temperatures, not only for tenecteplase but all drugs carried by their paramedics, due to the impact it could ultimately have on patient wellbeing.

5.2.3. STUDY C – The stability of EBC-46

The findings of this study will provide valuable information to researchers and stakeholders on the most appropriate storage conditions to ensure maximum stability of EBC-46, as well as where metabolism could occur *in vivo* and the degradation products that may be produced. Further to this, they indicate the conditions that result in EBC-46 degradation and how specific metabolites are acquired, which could be useful information for future research on these degradation products. Gathering this stability data is an important step in the development of a drug; it may not only affect how researchers prepare the pure product for experimental purposes but could have implications on formulation considerations as clinical trials progress. Consequently, the stability information obtained during this study will serve to increase the suitability of EBC-46 as a widely used treatment

for solid tumours and non-healing wounds, as it will ensure these properties are preserved for as long as possible during storage.

5.3. Limitations and future directions

5.3.1. STUDY A – The stability of remifentanil and propofol in combination

An obvious future direction for this study is to test a variety of anaesthetic and analgesic combinations that could benefit from simultaneous administration. However, there are aspects of the remifentanil and propofol combination that warrant additional research. For example, the effect of pH on the degradation of remifentanil, propofol, and a mixture of the two, and whether manipulating it might improve their stability, could be explored further. This may involve utilising various buffers to increase the scope of pH values tested, allowing for a comprehensive assessment of its influence. For example, a more precise pH range that induces remifentanil hydrolysis, and how the addition of propofol impacts this, could be determined. In addition to further pH work, emulsion globule size and distribution studies could be undertaken to give a better indication of possible interactions that may be occurring when propofol is mixed with remifentanil and different diluents. Relating to the findings from our study, 20% saline solution would be of interest for these tests as its addition typically induced the greatest propofol degradation, both in isolation and after mixing with remifentanil. As preliminary findings indicate that saline may have caused some propofol emulsion breakdown, it would also be pertinent to investigate other moderately acidic injection solutions and determine if their addition had similar effects. For example, glucose 5% intravenous infusion BP is a diluent recommended by both remifentanil and propofol manufacturers and has a pH of 3.5 – 6.5 (Baxter Healthcare Ltd 2018), while 5% dextrose injection is a commonly-used intravenous solution that has a pH of 4.3 (3.2 – 6.5) (Hospira Inc. 2005). Saline solution could also be removed altogether by adding lyophilised remifentanil directly into the propofol emulsion to prepare the mixture. Moreover, the original studies could be repeated using a prepared propofol solution that has the lipid removed, to explore how the stability of the mixture is affected without this excipient. Finally, the biological effects of any chemically-stable mixture should be scrutinised in *ex vivo* models before being trialled in patients.

5.3.2. STUDY B – The stability of lyophilised tenecteplase at high temperatures

Studies concerning tenecteplase stability predominantly assess the reconstituted solution; lyophilised tenecteplase is rarely investigated, despite the increased relevance to

paramedics and emergency clinicians. As such, it is considered essential that more studies focus on the degradation of tenecteplase in its solid form. Firstly, it would be beneficial to repeat the current study to increase the sample size, as the very high cost of tenecteplase meant only two samples per temperature treatment were possible. Following this, small variations could be made to the methodology to help fill current gaps in knowledge and provide a more comprehensive picture of how ambulance storage affects lyophilised tenecteplase stability. For example, the drug could be subjected to several eight-hour sessions of high temperature with controlled room temperature storage in between, simulating many consecutive days of vehicle storage. Alternatively, shorter, more frequent cycles of controlled room temperature and heat could be tested over an extended period, as this may be a more accurate representation of real-world scenarios. Furthermore, while it has been investigated using reconstituted tenecteplase, the effect of freeze/thaw cycling on lyophilised tenecteplase stability has, to our knowledge, not yet been studied, and could potentially be a means of preventing degradation in hot environments. Eventually, it would be valuable to perform *in vivo* studies using heat-exposed samples, to determine if *in vitro* efficacy findings are translated. Simulating a clot retraction *in vivo* and knowingly providing defective thrombolytics to a human infarct patient is unethical. Therefore, this area of research would need to be further explored using animal models, despite a difference in coagulation profiles making extrapolation to a human thrombosis model difficult. Finally, it would be interesting to explore other potential causes of tenecteplase degradation beyond temperature, and assess possible methods of prevention. This could include reconstituting tenecteplase with a variety of diluents or manipulating solution pH (with reference to preliminary data obtained from the current investigation).

5.3.3. STUDY C – The stability of EBC-46

For several reasons, the future directions of this study mostly involve expansion of the current research project to improve analytical outcomes. The biggest limitation of this study was the sample size, with a maximum of two samples per experiment. This was mostly due to the cost and limited availability of the EBC-46 pure product; understandably, priority of sample usage was given to other projects being conducted at the QIMR Berghofer Medical Research Institute. While sample size was not ideal for any of the experiments in this study, the lack of numbers was particularly evident for the air, nitrogen, and kidney tissue tests. Each of these only had one sample available, and all had conflicting results that made conclusions difficult to draw; both the air and nitrogen samples were found to simultaneously contain the greatest concentrations of EBC-46 and metabolite EBC-211, while the kidney tissue samples showed no change in EBC-46 concentration but a large increase in EBC-211. It is possible these unexpected findings may have been the result of

prior degradation in the EBC-46 master stock that was used to prepare all samples in this study, as this solution had been made and stored at 4°C for a time prior to study commencement. There would be great value in preparing fresh master stock and repeating not only these, but all experiments, as it would increase the accuracy of the findings and allow more solid conclusions to be deduced. Further to this, the light experiment should be performed using a UV light rather than natural sunlight, as this would eliminate heat as a contributing factor to any degradation observed and allow a narrow wavelength to be tested, and should also be performed under oxygen-free conditions (using inert gas and degassed solvents) so as to separate photochemical degradation from photochemically-induced oxidation. The nitrogen-treated solutions should also be prepared with degassed solvents to ensure all oxygen is removed from the sample. Regarding the pH experiments, there are a number of modifications that may lessen possible buffer-induced changes to EBC-46 degradation. For instance, to avoid using different buffers, one buffer with a wide pH range (such as the Prideaux-Ward or Carmody buffers, both with a range of 2 – 12 (Prideaux & Ward 1924; Carmody 1961)) could be used. Additionally, testing different buffers with the same pH and ionic strength, and testing one buffer at one pH/ionic strength but altering the concentration, could also determine if buffer salts have an effect on EBC-46 degradation; this information could then be used to construct a pH-rate profile for EBC-46. Another suggested modification is to perform the *ex vivo* experiments (i.e. liver and kidney tissue metabolism) at physiological temperature and pH, as an authentic representation of *in vivo* conditions might more accurately indicate possible degradation patterns in humans. The temperature study could also be expanded upon by assessing the impact of freeze-thaw cycles on the pure product. Furthermore, it would be valuable to test the compatibility of EBC-46 with a range of infusion agents, such as 5% dextrose/glucose and Ringer's lactate solution, as their use may be necessary for successful patient administration in the future. The effect of ionic strength on the hydrolysis of the EBC-46 epoxide to EBC-158 could also be investigated further by mixing with 20% saline solution. Finally, while a temperature of 4°C and solution pH of 5 were deemed the most suitable conditions for the short-term (i.e. one month) storage of the EBC-46 pure product, it would be interesting (and possibly necessary) to determine how long it would remain stable in this environment. This would require intermittent sampling and analysis, with the minimum potency level that is generally recognised as acceptable being 90% of the initial concentration (Kommanaboyina & Rhodes 1999). Its efficacy should also be examined concurrently using appropriate rodent tumour models to ensure this has not been affected, irrespective of concentration changes.

5.4. Conclusion

This investigation had two predominant outcomes. Firstly, it discussed the direct impact storage conditions can have on the stability and efficacy of a range of pharmaceuticals and how this may compromise patient wellbeing. Secondly, it considered how a drug's storage conditions could be modified to conceivably mitigate the rate of degradation, such as increased vigilance in following existing protocols or manipulating physical/chemical factors to provide a more suitable storage environment. While the studies examined specific drugs, the methodology and findings are very likely applicable to similar pharmaceuticals, particularly in the remote/rural emergency medicine setting, and there is potential for additional studies to be developed based on the concepts explored. Because of this capacity for further implementation, the knowledge gathered from this investigation may be of interest to a wide variety of clinicians and researchers within the pharmaceutical discipline and could elicit improvements in the storage and administration of numerous drugs.

The findings of these studies demonstrate a significant association between the storage environment of pharmaceuticals and the outcome of patients receiving them. As patient safety is paramount, the stability of drugs outside of the body should be considered a crucial aspect of pharmaceutical research that warrants continuous investigation.

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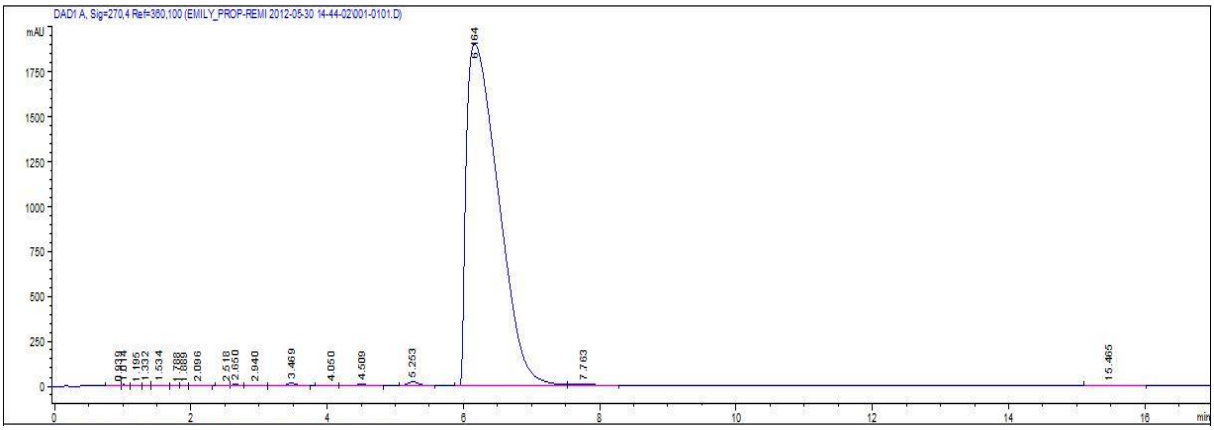
7. APPENDIX

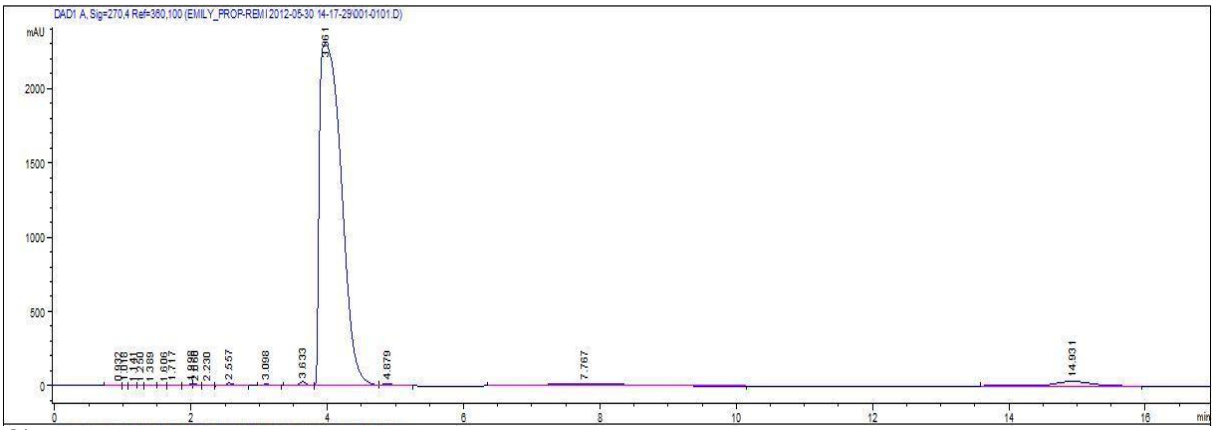
7.1. STUDY A – The stability of remifentanil and propofol in combination

7.1.1. Method development

A)



B)



C)

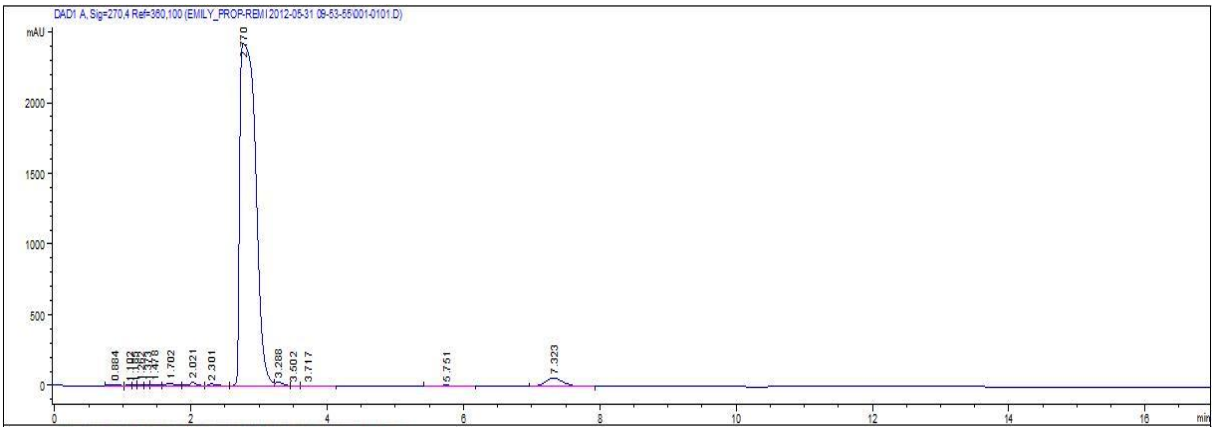
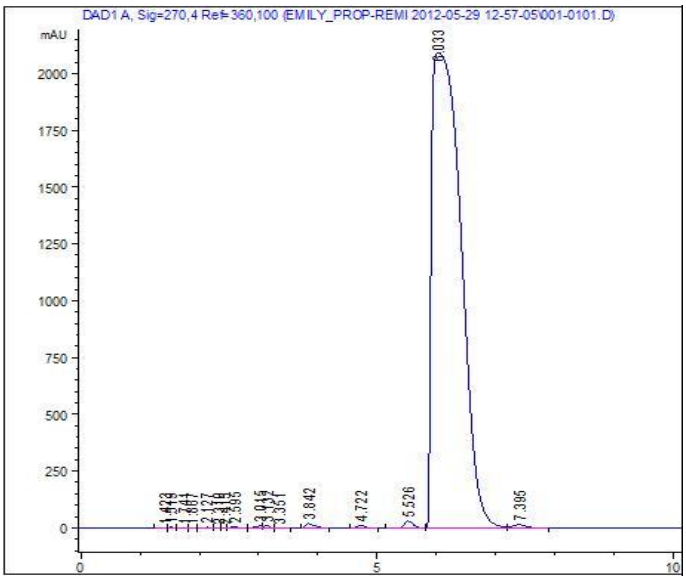


Figure 7.1. Analysis of a propofol solution using a mobile phase ratio of A) 65:35; B) 70:30; and C) 75:25. Increasing the amount of mobile phase A resulted in a narrower peak with a smaller retention time.

A)



B)

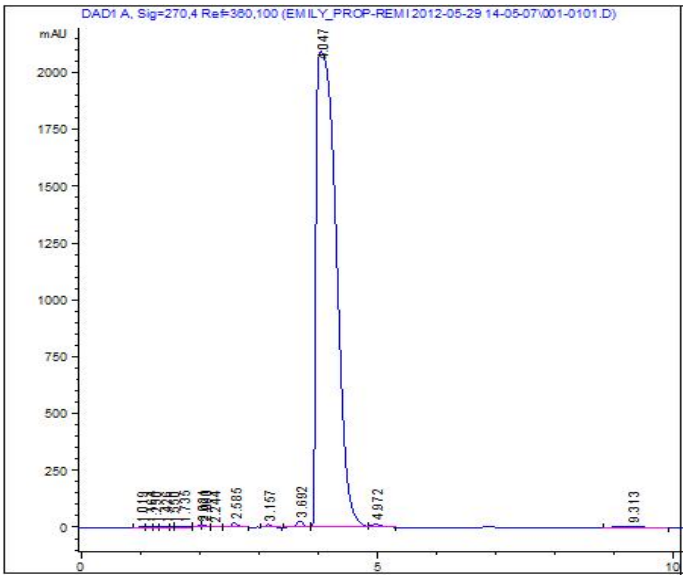
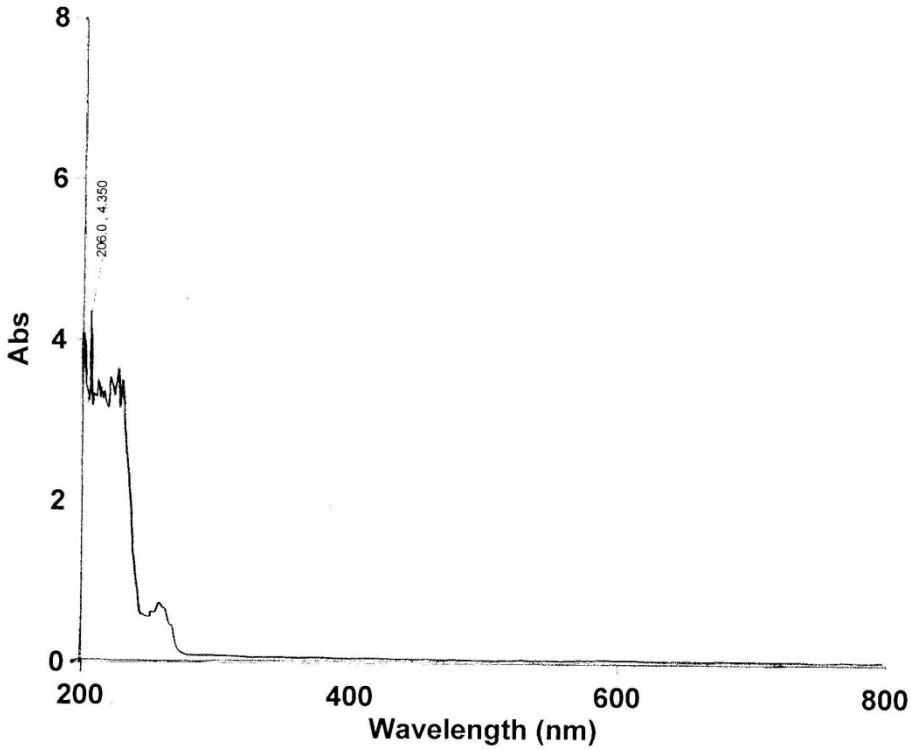


Figure 7.2. Analysis of a propofol solution using a mobile phase flow rate of A) 1 mL/min, and B) 1.5 mL/min. Increasing the flow rate resulted in a narrower peak with a smaller retention time.

A)



B)

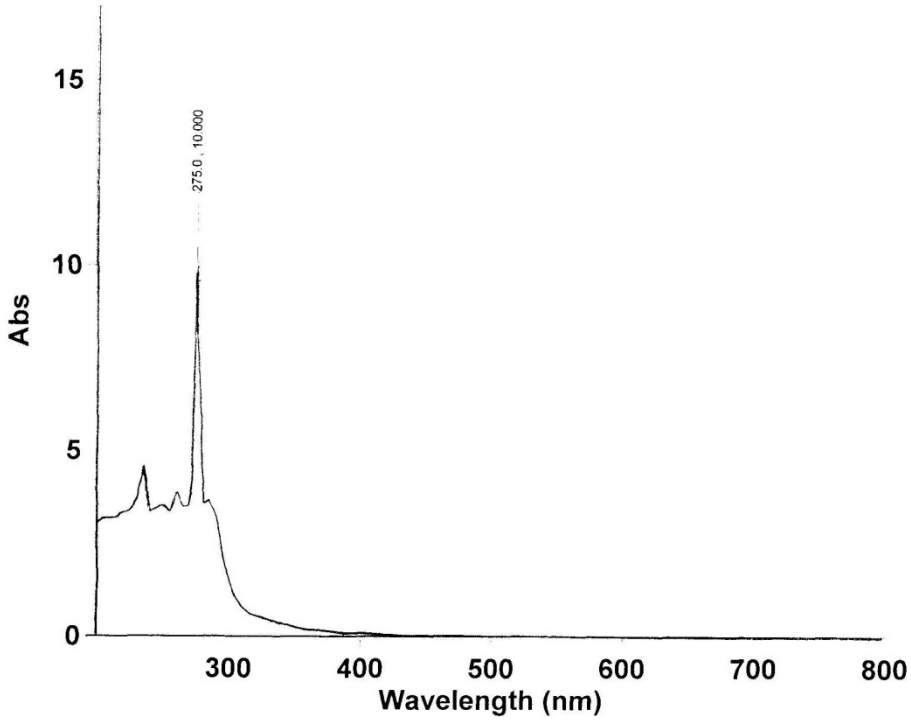


Figure 7.3. Spectrophotometric scans of A) a reconstituted remifentanil solution, and B) a filtered propofol solution. Maximum absorption is at a wavelength of 206 nm for remifentanil and 275 nm for propofol.

7.1.2. Additional method information

Diagrammatic representations of remifentanil (Figure 7.4) and propofol (Figure 7.5) sample preparations have been included below. Remifentanil concentrations of 10, 20, 30, 40 and 50 µg/mL were chosen for this study as: 1) the recommendation from remifentanil manufacturers is that it be diluted to a final concentration of 20, 25, 50 (the recommended dilution for adults), or 250 µg/mL prior to administration (Ultiva (remifentanil hydrochloride) for Injection Product Information 2011); 2) concentrations between 10 – 50 µg/mL can be easily prepared, as remifentanil is available in vials containing 1, 2 and 5 mg of the lyophilised drug; and 3) they provide a suitable range that allows trends between concentration and degradation to be observed. The initial propofol concentration for all solutions was the same, i.e. 10 mg/mL in 100 mL as originally supplied in the bottle, with final propofol concentration determined by the amount of reconstituted remifentanil or diluent that was added (e.g. adding 5 mL of diluent to 100 mL of 10 mg/mL propofol results in a final propofol concentration of 9.5 mg/mL).

Remifentanil samples analysed in isolation enabled the effect of diluent and pH on remifentanil degradation to be determined, while mixing remifentanil with propofol prior to analysis allowed the effect of propofol on remifentanil degradation to be observed. Propofol samples analysed in isolation enabled the effect of diluent and pH on propofol degradation to be determined, while mixing propofol with remifentanil prior to analysis allowed the effect of remifentanil on propofol degradation to be observed.

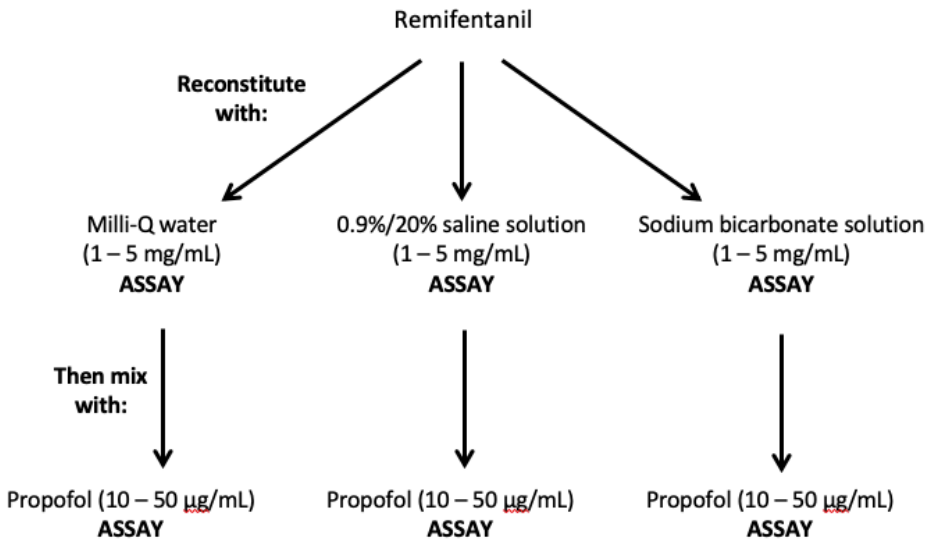


Figure 7.4. Diagrammatic representation of remifentanil methods.

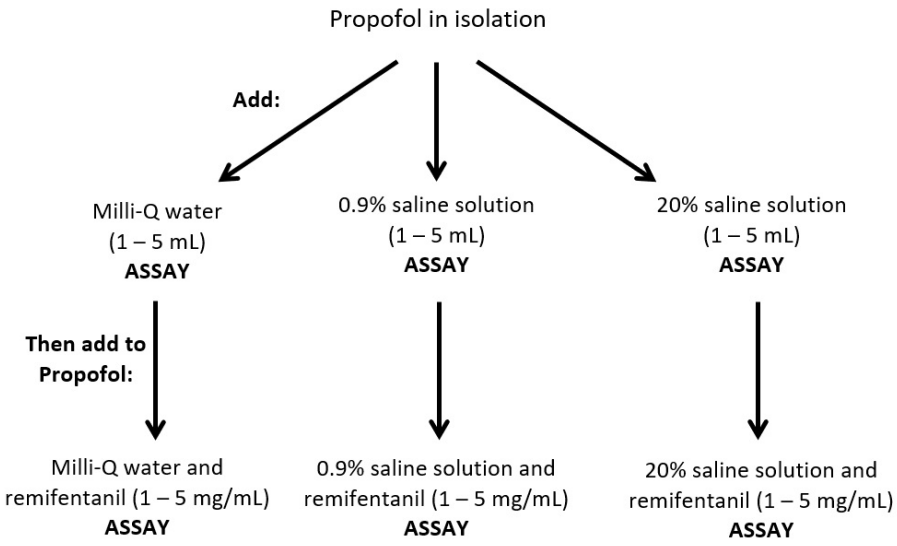


Figure 7.5. Diagrammatic representation of propofol methods.

7.2. STUDY B – The stability of lyophilised tenecteplase at high temperatures

7.2.1. Calculation of the mean kinetic temperature for the preliminary study

According to Kommanaboyina & Rhodes (1999), "the mean kinetic temperature (MKT) is the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures." It is commonly referred to in studies as it takes into account the duration of fluctuations in storage temperature, and the degree to which these would impact upon degradation. The Haynes' equation is typically used to calculate MKT. The equation used for this study specifically was as follows:

$$MKT = \frac{\Delta H/R}{-\ln((e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + e^{-\Delta H/RT_3})/n)}$$

where ΔH is the energy of activation (83.144 kJ/mol), R is the universal gas constant (0.0083144 kJ/mol/degree), T_1 is the average of the highest and lowest temperatures (in degrees Kelvin, K) recorded during the first time period (i.e. the first day), T_2 is the average of the highest and lowest temperatures recorded during the second time period (i.e. the second day), T_3 is the average of the highest and lowest temperatures recorded during the third time period (i.e. the third day), and n is the total number of average storage temperatures recorded.

7.2.2. Synthesis of arginine phosphate

One component of the buffer required for the tenecteplase concentration assay, arginine phosphate, is not available commercially so had to be synthesised. The method used to achieve this was adapted from Kim (2000), Premkumar, Shajan & Devadoss (2010) and Muley et al. (2014). Briefly, equimolar quantities of L-arginine and phosphoric acid were mixed thoroughly using a magnetic stirrer at 45 - 50°C for 4 hours, filtered five times with membrane filters, and evaporated to form crystallised arginine phosphate. L-arginine was purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia), and phosphoric acid was purchased from Astral Scientific Pty Ltd (Taren Point, NSW, Australia).

7.2.3. D-dimer assay

While a general description of the D-dimer assay was provided in Chapter 1 (see section 1.2.3.6.), a more detailed explanation of the specific D-dimer assay used in this investigation (see Chapter 3), including the method for sample preparation, is included here.

7.2.3.1. D-dimer monoclonal antibody

The assay, INNOVANCE D-Dimer, employs polystyrene particles coated with the 8D3 monoclonal antibody (mAb) (Innovance D-Dimer Package Insert 2017). Like all mAb for D-dimer, 8D3 are created using a standard technique. Briefly: the D-dimer antigen is prepared by lysing fibrinogen; mouse spleen cells are immunised with the D-dimer antigen; and the immunised cells are fused with myeloma cells, creating a hybridoma cell line that secretes D-dimer-specific mAb (Rylatt et al. 1983; Holvoet et al. 1989; Kogan et al. 2016). Regarding specificity, the 8D3 mAb has been shown to react with fragment D-dimer of crosslinked fibrin and fragment D (a plasmin-resistant or terminal core fragment produced by fibrin breakdown) of non-crosslinked fibrin (Gaffney 1980; Holvoet et al. 1989).

7.2.3.2. INNOVANCE D-Dimer

The INNOVANCE D-Dimer kit includes reagent (containing the polystyrene particles coated with mAb to D-dimer), buffer, supplementary reagent, sample diluent, and a calibrator fluid, all of which require some form of preparation (i.e. reconstitution, removal of bubbles, resuspension of precipitates) prior to use. This is outlined in the INNOVANCE D-Dimer Package Insert (2017). Following this, the plasma sample can be assayed. Briefly, diluent, buffer, and supplementary reagent is added to the plasma sample and incubated at 37°C for 3 minutes. The reagent is then added, inducing agglutination of any D-dimer present around the mAb-coated polystyrene particles (Herak, Milos & Zadro 2009;

Innovance D-Dimer Package Insert 2017). The concentration of D-dimer is directly proportional to the degree of agglutination, determined by subjecting the sample to light at a wavelength of 575 nm and measuring the decrease in transmittance using a coagulation analyser (in this instance, a Sysmex CA-560) (Sysmex CA-560 Brochure 2008; Herak, Milos & Zadro 2009). The measuring range of the INNOVANCE D-Dimer assay is given as 0.17 – 4.40 mg/L fibrinogen equivalent units (FEU), with samples measuring outside this range automatically diluted (1:8) to extend the range to 35.2 mg/L FEU (Innovance D-Dimer Package Insert 2017). FEU are based on the molecular weight of fibrinogen (320 – 340 kD) that would have been present initially to create the fibrin degradation products detected in the assay, and are approximately 1.7 times the molecular weight of fibrin fragment D-dimer (Dempfle 2005).

7.3. STUDY C – The stability of EBC-46

7.3.1. Metabolite concentration determination

Where metabolite standards were not analysed concurrently with samples, their concentration was calculated based on EBC-46 chromatogram results using the following equation:

$$\text{Metabolite conc. (mg/mL)} = \left(\frac{\text{area of metabolite peak}}{\text{area of EBC-46 peak}}\right) \text{x conc. EBC-46 (mg/mL)}$$

7.3.2. Additional method information

7.3.2.1. The effect of temperature, light and air

The following diagrammatic representations demonstrate the method used to determine temperature, light and air effects on EBC-46 stability (Figure 7.6), and the experimental setup used for the “air” trial (Figure 7.7).

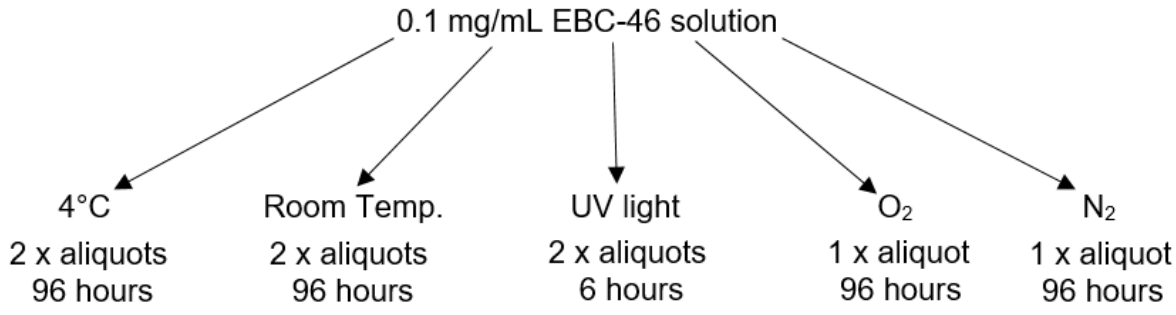


Figure 7.6. Diagrammatic representation of temperature, light and air method

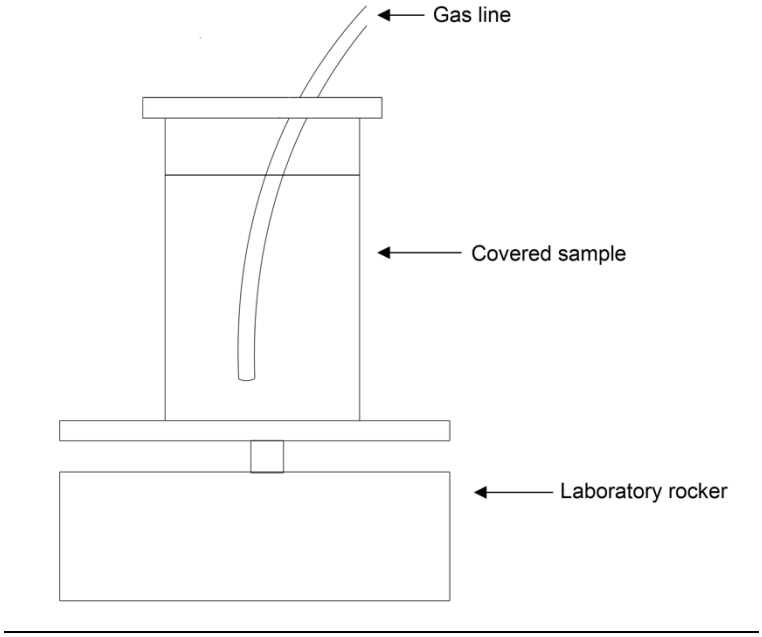


Figure 7.7. Experimental setup of “air” trial

7.3.2.2. The effect of pH and temperature

The buffers used to adjust solution pH were as follows:

- pH 3 – 6: 0.01 M acetate buffer, containing acetic acid and sodium acetate
- pH 6 – 8: 0.01 M phosphate buffer, containing sodium dihydrogen phosphate and sodium hydrogen phosphate
- pH 8 – 9: 0.01 M borate buffer, containing boric acid and sodium tetraborate

After preparing 0.01 M solutions of the acids and conjugate bases, the ratio of each needed to make the required pH values was determined using the Henderson-Hasselbalch equation, as follows:

$$pH = pK_a + \log_{10}\left(\frac{[A^-]}{[HA]}\right)$$

Where pK_a is the negative log of the acid dissociation constant (4.76 for acetic acid, 6.82 for sodium dihydrogen phosphate, and 9.15 for boric acid), [HA] is the concentration of the acid, and $[A^-]$ is the concentration of the conjugate base.

The following table shows the amount of acid and base used to prepare 100 mL buffers for each pH value:

Table 7.1. Volume of acid and base required for EBC-46 buffers

Acetate Buffer		
pH	**0.01 M Acetic Acid (mL)**	**0.01 M Sodium Acetate (mL)**
3	98.2	1.8
4	84.7	15.3
5	35.7	64.3
6	5.2	94.8
Phosphate Buffer		
pH	**0.01 M Sodium Dihydrogen Phosphate**	**0.01 M Sodium Hydrogen Phosphate**
6	87.7	12.3
7	39.0	61.0
8	5.3	94.7
Borate Buffer		
pH	**0.01 M Boric Acid**	**0.01 M Sodium Tetraborate**
8	94.6	5.4
9	63.5	36.5

During buffer preparation, the pH was monitored using a pH meter. If required, acid or base was added until the desired pH was achieved.