DEGRADATION OF PARACETAMOL AND OTHER CONSTITUENTS IN PERFALGAN®

by

DR CATHERINE CURRAN

(née JACKSON)

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SUPERVISOR: PROFESSOR DAVID MORRELL

Head of Department of Anaesthesia, East London Hospital Complex

ASSOCIATE SUPERVISORS:

ASSOCIATE PROFESSOR NEIL RAVENSCROFT and PROFESSOR GRAHAM JACKSON

Department of Inorganic Chemistry, University of Cape Town

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3 PLAGERISM DECLARATION

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4 DISCLAIMER

This is a non-clinical study which analysed Perfalgan® over time and under various conditions. The findings were not intended to challenge the prescribing guidelines set out by the manufacturers, Bristol-Myers Squibb. As always, practitioners are advised to use their clinical discretion when administering this, and any, drug.

5 SUMMARY

5.1 Background

Paracetamol (acetaminophen) is useful for the treatment of mild to moderate pain as well as being opiate–sparing. In recent years the problem of poor solubility of paracetamol in water has been overcome and an intravenous formulation of paracetamol developed. In South Africa this is marketed by Bristol-Myers Squibb as Perfalgan®. Each 500 mg or 1g ampoule of Perfalgan® is recommended for single use only and is to be discarded once opened (Medicines.org, n.d.). This is most likely due to concerns about degradation of paracetamol or other ingredients in the solution once exposed to air and sterility issues. However in South Africa where this drug is expensive, some centers use one ampoule for multiple cases over the course of 12-24 hours. No obvious clinical adverse effects have been reported.

5.2 Objective of study

The aim of this study was to examine the practice of using a single vial of Perfalgan® in divided doses over the course of a day for different patients by way of assessing the stability of Perfalgan® on exposure to air.

The study has three components, namely:

1. *In vitro* assessment of paracetamol levels on exposure to air and stressors over time.
2. *In vitro* assessment of levels of the excipients, namely mannitol and cysteine on exposure to air and stressors over time.
3. Assessment of the lipid permeability of paracetamol in Perfalgan® on exposure to air and stressors over time.

5.3 Methodology

High performance liquid chromatography (HPCL-UV) was used to determine the concentration of paracetamol and the presence of degradation products in samples taken at set time periods following exposure of Perfalgan® to air and stressors.

Initial work was done using pure paracetamol to determine optimal measurement conditions prior to analysing Perfalgan®.

The concentrations of the additives and their degradation products were measured using nuclear magnetic resonance (^{1}H NMR). Appreciable changes in these could indicate a safety hazard or decreased bioavailability.

Finally ultraviolet spectroscopy was performed to assess samples at a wider spectrum of wavelengths, instead of the single wavelength used in HPLC. This was used to assess for degradation products which absorb at different wavelengths and therefore may be missed if HPLC was used alone. Ultraviolet spectroscopy was also used to test bioavailability of the drug via the octanol:water partition coefficient under stressed and unstressed conditions.

5.4 Results

The paracetamol in Perfalgan® did not degrade on exposure to air over 24 hours. Neither did it degrade on exposure to acid, alkali, oxidative or heat stress. The HPLC retention time was constantly between 3.17-3.29 minutes. ^{1}H NMR revealed no change in the formulation of Perfalgan® except for the conversion of the oxygen scavenger cysteine to cystine. The octanol:water partition coefficient likewise stayed constant and was in agreement with the value of 0.46-0.49 quoted in the literature (International Programme on Chemical Safety, 2008; McNeil Consumer Healthcare, 2010).

5.5 Conclusion

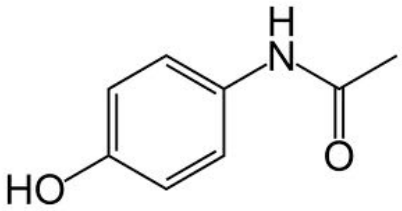
The paracetamol in Perfalgan® did not degrade on exposure to air and other stressors over 24 hours. The formulation as Perfalgan® was likewise found to be stable. The drug retained its lipid permeability over this period.

6 INTRODUCTION AND LITERATURE REVIEW

6.1 Paracetamol

6.1.1 Physical and chemical properties

Paracetamol or N-(4-hydroxyphenyl)acetamide consists of a benzene ring core, substituted with one hydroxyl group and an amide group (Bales, et al., 1985). It is known as acetaminophen in the United States of America.



Physical and Chemical Characteristics: (O'Neil, 2006)

Molecular formula: C_8-H_9-N-0_2

Molecular weight: 151.16

Odourless with a slightly bitter taste

Melting point: 169-170.5 °C

Density: 1.293 at 21 ^{o}C

Dissociation constant: pKa = 9.38

pH of a saturated aqueous solution: 5.5-6.5

Paracetamol is poorly soluble in cold water, but more so in hot water. One part of paracetamol is soluble in 70 parts of water at room temperature, and 1 in 20 parts in boiling water. The aqueous solubility is elsewhere reported as 14.7 mg.ml^{-1} at 20 °C, 14.3 mg.ml^{-1} at 25 °C and 23.7 mg.ml^{-1} at 37 °C (Kalantzi, Reppas and Dressman, 2006). It is soluble in methanol, ethanol,

dimethylformamide, ethylene dichloride, acetone and ethyl acetate. It is slightly soluble in ether and practically insoluble in petroleum ether, pentane and benzene (O'Neil, 2006).

6.1.2 History

The analgesic and antipyretic properties of paracetamol were first discovered in the late nineteenth century. Two agents were already in use for the treatment of mild to moderate pain, acetanilide and phenacetin. In 1889 it was shown that paracetamol was excreted in the urine of patients who had taken these drugs. In 1948 it was established that paracetamol was the major metabolite of both phenacetin and acetanilide. This led to the belief that the clinical effects of these two drugs were entirely due to the rapid conversion to paracetamol. It was later shown that phenacetin had some activity in its own right, but only at very high doses, as a great proportion is converted to paracetamol. However, by this stage phenacetin was believed to have greater toxicity than paracetamol due to somewhat circumstantial evidence of its role in causing analgesic nephropathy. Phenacetin disappeared from the market as paracetamol's popularity grew. In 1956 500 mg tablets became commercially available in the United Kingdom as a prescription drug, and in 1963 as an over-the-counter drug. It was subsequently combined with a variety of compound analgesics to provide multi-modal analgesia (Clissold, 1986; Brandon, n.d.).

6.1.3 Function and mechanism of action

Paracetamol has analgesic and antipyretic properties and weak anti-inflammatory activity. It is indicated for management of mild to moderate pain and fever (Rossiter, 2014). It has been used in the perioperative period because of its opioid-sparing effect.

The mechanism of action is not clearly elucidated. Most agree that paracetamol is active centrally, an impression supported by the fact that paracetamol is found in significant concentrations in cerebrospinal fluid of both adults and children after infusion (Woo, 2008). Theories of action include:

- Activation of descending serotonergic pathways which modulate pain perception. Allouia *et al* supported this theory by showing that administering a 5-HT3 antagonist blocks the pain-modulating activity of intrathecal paracetamol (Allouia, Chassaing and Schmidt, 2002).
- Inhibition of central prostaglandin synthesis by inactivating (reducing) the active (oxidized) form of cyclo-oxygenase (Oscier and Milner, 2009).
- Stimulation of endogenous cannabinoid receptors. Paracetamol is metabolized to AM404 (also known as N-arachidonoylphenolamine) which prevents the reuptake of endogenous cannabinoids from the synaptic cleft. This theory has gained popularity since Ottani *et al* showed that the blockade of the cannabinoid type 1 (CB1) receptors attenuated the action of paracetamol (Ottani, Leone and Sandrini, 2006; SAJAA, 2010).

With respect to the anti-inflammatory properties, paracetamol does not significantly affect peripheral cyclo-oxygenase. It is likely that paracetamol is most effective at inhibiting cyclo-oxygenase in environments such as the brain where peroxide concentration is low. Peripherally, especially in the setting of inflammation, peroxide concentration is high and the action of paracetamol on cyclo-oxygenase is greatly reduced. (Oscier and Milner, 2009) This accounts for its limited anti-inflammatory effects.

6.1.4 Dosing

There is a wide variety in dose recommendations in the literature. According to Rossiter (2014) the recommended dose of paracetamol is:

a) Adults:
0.5-1 g 4-6 hourly (maximum 4 $g.day^{-1}$)
Extended-relief formulation: 650-1300mg 8 hourly (maximum 3.9$g.day^{-1}$)

b) Children >one month:
Oral: 20 $mg.kg^{-1}$ 6 hourly (maximum 90 $mg.kg^{-1}.d^{-1}$)
Rectal: 20$mg.kg^{-1}$ 6 hourly (maximum 90 $mg.kg^{-1}.d^{-1}$). Loading dose of 40$mg.kg^{-1}$ permissible as long as total daily dose is adhered to.

Alternatively, according to the manufacturers, dosing can be adjusted by age. The following doses are recommended 4-6 hourly with a maximum of 4 doses in 24 hours.

Table 1: Paracetamol dosing according to age group

Age group	Oral	Rectal
3-12 months	60-120mg	60-125mg
1-5 years	120-240mg	125-250mg
6-12 years	250-500mg	250-500mg

c) Term neonates: 20 $mg.kg^{-1}$ 8 hourly (maximum of 60 $mg.kg^{-1}.d^{-1}$)

d) Premature neonates: 15$mg.kg^{-1}$ 8 hourly

At these doses it has an excellent safety profile, notably lacking the gastrointestinal side effects of aspirin and most non-steroidal anti-inflammatory agents. Rectal absorption is slower and more variable than oral dosing. Rectal administration of 25$mg.kg^{-1}$ to children achieved an average C_{max} at the lower end of the therapeutic range. Studies involving adults showed that only those with initial doses of 35-45$mg.kg^{-1}$ reached the therapeutic plasma concentration of 10 $ug.ml^{-1}$ (Oscier and Milner, 2009). An initial larger dose should therefore be given.

6.1.5 Pharmacokinetic properties

6.1.5.1 Absorption

Absolute bioavailability in the fasted state ranges between 62%-89%. Peak concentrations are reached within 0.17 to 1.2 hours. The presence of food in the stomach delays gastric emptying. This decreases the absorption of paracetamol thereby increasing the time to peak effect (t_{max}) and decreasing the maximum concentration (C_{max}) reached (Kalantzi, et al., 2006).

6.1.5.2 Distribution

The apparent volume of distribution of paracetamol is 0.69–1.36 $l.kg^{-1}$. Plasma protein binding is 20%–25% at therapeutic doses. After overdosage, 20% – 50% of the drug may be protein bound. Paracetamol crosses the placenta and into breast milk, where 85% is bound to milk proteins (Kalantzi, et al., 2006).

6.1.5.3 Degradation

6.1.5.3.1 In vitro

Paracetamol degradation *in vitro* occurs through 2 pathways. It is either degraded by oxidation to quinone-imines or by hydrolysis of the amino group generating p-aminophenol. P-aminophenol is quickly degraded producing p-benzoquinoneimine. Deacetylation takes places both under acid and (much faster) basic conditions (Nguyen-xuan, 2006).

6.1.5.3.2 In vivo metabolism and elimination

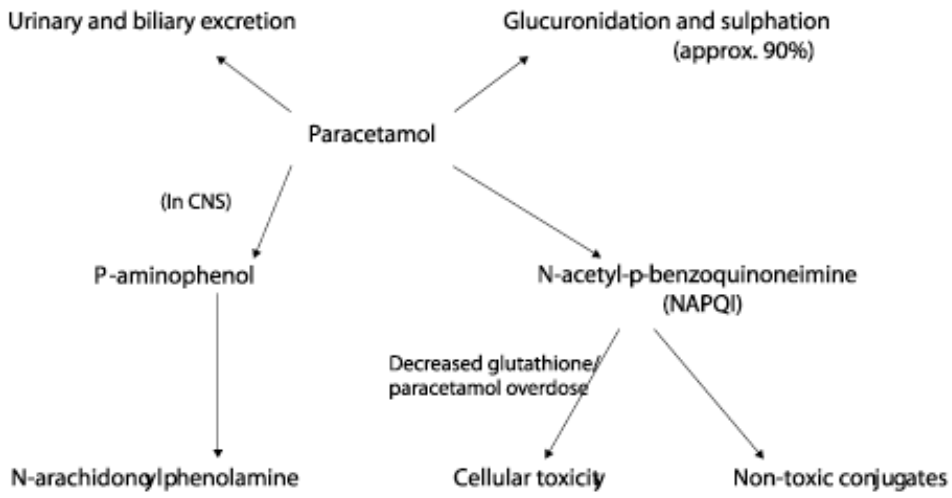


Figure 1: Paracetamol metabolism and elimination in vivo (Oscier and Milner, 2009)

In vivo, paracetamol is metabolised primarily in the liver into non-toxic, inactive products via three metabolic pathways:

1. Glucuronidation is believed to account for 40%-66% of the metabolism of paracetamol.
2. Sulphation (sulphate conjugation) may account for 20–40%.
3. N-hydroxylation and conjugation to glutathione accounts for less than 15%. The hepatic cytochrome P450 enzyme system (specifically CYPA2 and CYP2E1, and to a lesser extent CYP2D6) metabolizes paracetamol. A minor yet significant alkylating metabolite known as N-acetyl-p-benzo-quinoneimine (NAPQI) is formed. NAPQI is then irreversibly conjugated with the sulfhydryl groups of glutathione to form mercapturic acid conjugates and cysteine. (Oscier and Milner, 2009)

All three pathways yield final products that are inactive, non-toxic, and eventually excreted by the kidneys (Nelson, Flomenbaum and Goldfrank, 2006). Should NAPQI accumulate, significant hepatic and renal toxicity may occur, as discussed below with other adverse effects of paracetamol.

6.1.5.4 Elimination

Elimination is renally mediated but since only 5% of the drug is eliminated unchanged in the urine, patients with renal failure rarely have a prolonged drug effect (Woo, 2008). Plasma clearance is between 11.8-22.3 $l.hr^{-1}$. The elimination half-life is reported to be between 1.9 and 4.3 hours (Kalantzi, et al., 2006). An increased dose interval of 8 hours is recommended in patients with severe renal impairment (GFR of less than $10ml.min^{-1}$) (Oscier and Milner, 2009).

6.1.6 Adverse effects

At therapeutic doses, adverse effects of paracetamol are few. Two systematic reviews have found that the incidence of side effects following paracetamol administration are not significantly different to those of a placebo (Oscier and Milner, 2009). Allergic skin rashes and chronic nephritis occasionally occur with prolonged use (Rang, Dale and Ritter, 2000). It is however advisable that a patient is well hydrated before paracetamol administration.

However, acute overdose can be extremely serious. This causes hepato- and occasionally renotoxicity via accumulation of NAPQI (see above). Ingestion of 10-15 g of paracetamol by an adult may cause severe hepatocellular necrosis. Doses of 20–25 g are potentially fatal (Kalantzi, et al., 2006). These effects occur when the liver enzymes that normally conjugate metabolites become saturated. According to the third pathway mentioned above, NAPQI accumulates when glutathione is depleted, leading to cellular necrosis (Rang, Dale and Ritter, 2000).

Patients who may be particularly at risk for toxicity are:

- Those with genetic polymorphisms of the CYP-450 gene. They are rapid metabolisers and therefore more prone to NAPQI production.
- Those taking drugs which cause CYP P450 induction, such as ethanol, phenytoin or rifampicin (Woo, 2008).
- Fasted patients as they have a decreased ability to conjugate paracetamol with glucuronic acid due to decreased hepatic carbohydrate reserves. This may lead to increased microsomal oxidation and therefore increased risk of hepatotoxicity (International Programme on Chemical Safety, 1998).

Neonates and young children display different metabolism to adults. Here the main pathway is sulphate conjugation rather than glucuronidation. The normal ratio of glucuronidation:sulphation (2:1) is achieved by 12 years. The cytochrome P450 isoenzyme responsible for creating NAPBQI is less active than in adults, which offers some protection against hepatotoxicity in children. However, glutathione can become depleted in malnutrition and chronic paracetamol therapy putting the child at risk of toxicity (SAJAA, 2010).

6.1.7 Analysing paracetamol

Multiple methods have been used to analyse paracetamol. Most research is now directed at finding a single method to analyse combination drugs, as paracetamol is often a component of

compound analgesic tablets. Liquid chromatographic methods have been most widely accepted for use in detecting paracetamol (Franeta, Agbaba and Eric, 2002; Shah, et al., 2011).

According to Shah *et al* (2011), a reversed phase high performance liquid chromatographic (RP-HPLC) method is best for the simultaneous analysis of paracetamol and lornoxicam in tablet dosage form. In this study, a Brownlee C-18, 5 µm column having 250×4.6 mm internal diameter in isocratic mode, with mobile phase containing 0.05 M potassium dihydrogen phosphate:methanol (40:60, v/v) was used. The flow rate was 1.0 ml.min^{-1} and effluents were monitored at 266 nm. The retention time of paracetamol was 2.7 min. The linearity for paracetamol was in the range of 5–200 µg.ml^{-1}. The method used by Shah *et al* was replicated in this study.

According to Franeta, Agbaba and Eric (2002), RP-HPLC with UV detection without prior derivatization can be used for direct simultaneous determination of acetylsalicylic acid, caffeine, paracetamol and phenobarbital in tablet form. A mixture of acetonitrile:water (25:75 v/v) adjusted to pH 2.5 by adding phosphoric acid was used as a mobile phase at a flow rate of 2.0 ml.min^{-1}. As a compromise, a wavelength of 207 nm was used to detect these products. The graph below indicates the chromatograms for each substance.

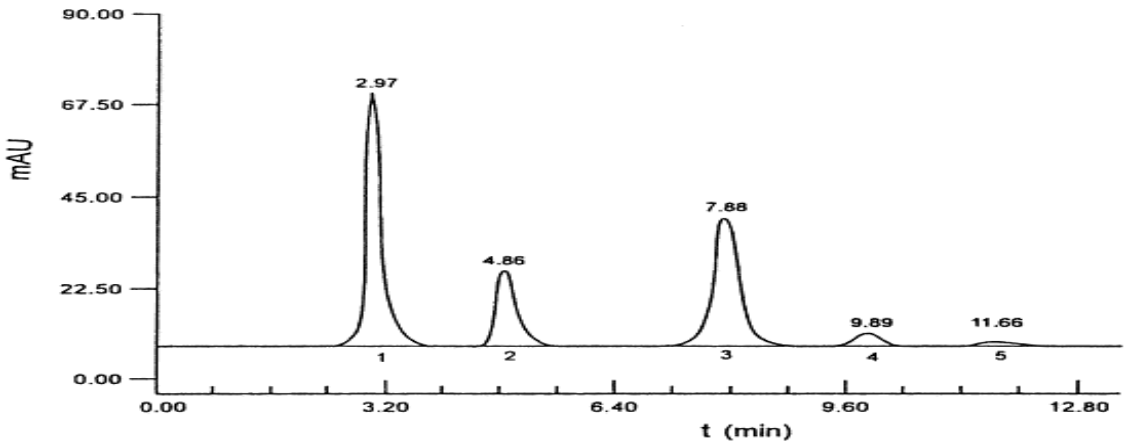


Figure 2: Chromatograms of acetylsalicylic acid, paracetamol, caffeine, phenobarbital and salicylic acid obtained by HPLC

The tR measured are as follows: acetylsalicylic acid (7.88 minutes), paracetamol (2.97 minutes),caffeine (4.86 minutes), phenobarbital (9.98 minutes) and salicylic acid (11.66 minutes).

Gopinath *et al* (2007) used a simple, selective, rapid, precise and economical reverse phase HPLC method for the simultaneous estimation of paracetamol and aceclofenac. This study was carried out on a Hichrom C18 (25 cm×4.6 mm i.d., 5 µ) column with a mobile phase consisting of acetonitrile:20 mM phosphate buffer (pH 5.0) (60:40 v/v) at a flow rate of 0.8 ml.min^{-1}. Detection was carried out at 265 nm. Etoricoxib was used as an internal standard. The retention time of paracetamol was 4.75 minutes.

6.1.8 The challenge of creating intravenous paracetamol

A soluble form of paracetamol has long been desired. While oral paracetamol is the cheapest and easiest mode of administration, this is not an option for intraoperative use or in patients with bowel obstruction. Rectal administration is unreliable with erratic absorption and peak plasma concentration is variable and reached 2-3 hours later than by the oral route. For most drugs, intravenous dosing is the most reliable form of administration and peak plasma concentrations are reached more quickly and consistently than by any other route (Woo, 2008).

However, due to paracetamol's poor water solubility, until recently the only intravenous preparation was proparacetamol. This is a pro-drug of paracetamol which requires hydrolysis by plasma esterases to yield the active drug, paracetamol and byproduct, diethylglycine. A dose of 2g of proparacetamol is equivalent to 1g of paracetamol (Mohammed, et al., 2012). However, the risk of hypersensitivity and pain on injection limits use of proparacetamol. Also proparacetamol is less convenient as it requires reconstitution (Duggan and Scott, 2009).

6.2 Perfalgan®

In recent years, through the use of improved stabilization techniques, paracetamol was developed as an intravenous form, Perfalgan®. Hydrophilic ingredients like mannitol and disodium phosphate were added to make it water soluble (Woo, 2008). Unlike proparacetamol, this no longer needs the hydrolysis step for activation. In South Africa intravenous paracetamol is marketed by Bristol-

MyersSquibb as Perfalgan®.

6.2.1 Contents

Perfalgan® contains 10 mg.ml^{-1} of paracetamol (a 50ml vial contains 500 mg of paracetamol, a 100 ml vial contains 1 g of paracetamol). According to the manufacturers (Granberg and Rasmuson, 1999; Woo, 2008; Medicines.org, n.d.), other contents include:

a. mannitol: aids solubility
b. cysteine hydrochloride and nitrogen: preservatives and antioxidants
c. sodium phosphate – dibasic dehydrate: buffer (maintains pH of 5.5, minimizing hydrolysis)
d. sodium hydroxide: buffer
e. hydrochloric acid: buffer
f. water for injection

These additives are called excipients. Some of them can be analysed by nuclear magnetic resonance (NMR), as explained below.

With an osmolarity of 290 mOsm.l^{-1}, Perfalgan® offers little or no pain at the site of injection (Schmitt *et al*, 2003). It is packaged in a glass container with a rubber stopper as a clear or slightly yellowish solution. No indication is given as to the source of the yellow colour. The manufacturer's explanation of adding the sodium hydroxide and hydrochloric acid as buffers is misleading as these compounds do not form a buffer but react to form salt (NaCl) and water.

6.2.2 Storage and shelf-life

It is recommended that Perfalgan® should be stored below 30 °C. Refrigeration is not advised. Before administration, the vial should be inspected for discolouration or particulate matter (Bristol-Myers Squibb, 2013).

6.2.3 Administration

Perfalgan® should be infused over 15 minutes (Bristol-Myers Squibb, 2013). The manufacturer gives no reason for this. A slower infusion may give a lower peak effect but there is no indication that a slower infusion results in increased degradation within the vial and administration set. Each vial is advised for single use only. This is presumably due to concerns about degradation of the product and because the vial contains no antimicrobial agent. Perfalgan® 10 $mg.ml^{-1}$ solution can also be diluted in a 0.9% sodium chloride or 5% glucose solution up to one tenth. In this situation the manufacturers advise that the diluted solution should be used within one hour, including the infusion time (Medicines.org, n.d.). No clear reason for this is given. Common practice is not to dilute the drug, even for paediatric use.

6.2.4 Dosing

The manufacturer-advised dosing schedule for Perfalgan® is represented in Table 2 (Bristol-Myers Squibb, 2012).

Table 2: Perfalgan® dosing information

Patient Weight	**Paracetamol dose ($mg.ml^{-1}$) per administration**	**Maximum number of doses per day**	**Minimum interval between each administration**	**Maximum dose per 24 hours**
> 50 kg	1g (i.e. one 100 mL vial)	4	4 hours*	≤ 4g
33 kg - 50 kg	15 $mg.kg^{-1}$ (i.e. 1.5 ml solution per kg)	4	4 hours*	≤ 60 $mg.kg^{-1}$, without exceeding 3 g
10 kg - 33 kg	15 $mg.kg^{-1}$ (i.e. 1.5 ml solution per kg)	4	6 hours	≤ 60 $mg.kg^{-1}$, without exceeding 2 g
≤ 10 kg**	7.5 $mg.kg^{-1}$ (i.e. 0.75 mL solution per kg)	4	6 hours	≤ 30 $mg.kg^{-1}$

**The minimum interval between each administration must be 4 hours in patients without hepatic or renal impairment. However, in patients with renal and/or hepatic impairment the minimum interval between doses must not be less than 6 hours.*
 # The maximum daily dose takes into account all medicines containing paracetamol or proparacetamol.
*** No safety and efficacy data are available for premature neonates. There is limited data on the use of Perfalgan® in neonates and infants less than 6 months of age.*

In patients with severe renal impairment (creatinine clearance of less than 30ml.min^{-1}) the dosing interval should be increased to 6 hours. The elderly do not require dosage adjustment (Duggan and Scott, 2009). Recommendations for patients with liver disease vary according to the reference used. Duggan and Scott (2009) say no dosage adjustment is required. However the South African Perfalgan® package insert advocates a maximum dose of 3 g.day^{-1} in those with chronic or active hepatic disease, especially with hepatocellular insufficiency, chronic alcoholism, malnutrition and dehydration (Bristol-Myers Squibb, 2013).

6.2.5 Pharmacokinetics

6.2.5.1 Distribution

Intravenous paracetamol reaches its peak concentration as soon as the infusion is completed. According to the manufacturers, the effect starts within 5 minutes, peaking at 1 hour and lasting for up to 6 hours. The antipyrexial effect lasts slightly longer than the analgesic effect. The plasma half-life is 2.7 hours. Unlike other routes of administration, the speed of onset can be altered. If the infusion rate is slowed down, onset and t_{max} will be delayed. In large patients, the peak effect may be decreased as the higher volume of distribution (Vd) correlates with a lower peak plasma level.

6.2.5.2 Metabolism and elimination

Paracetamol follows the same metabolic and elimination pathways irrespective of mode of delivery. In severe renal failure (creatinine clearance 10-30ml.min^{-1}) the $t_{½}$ increases to 2.0-5.3 hours, so the dosing interval should be increased from 4 to 6 hours. The pharmacokinetics of paracetamol are not significantly changed in the elderly (Duggan and Scott, 2009).

6.2.6 Side effects

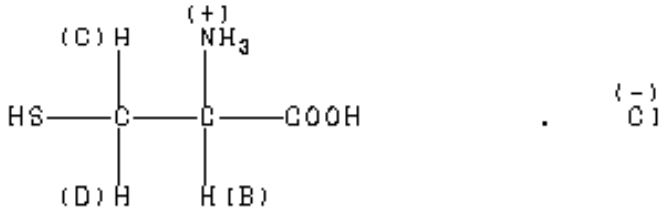
As with the oral formulation, side effects are rare at therapeautic doses. They include rashes, blood disorders and hypotension on infusion. The skin reaction at infusion site seen with proparacetamol is rarely seen with Perfalgan® (Duggan and Scott, 2009).

6.2.7 Excipients

(Yamaji, *et al*., 2012)

6.2.7.1 Cysteine hydrochloride:

6.2.7.1.1 *Structure*



This is a white crystalline powder soluble in water. The structural formula is $C_3H_7NO_2S$• HCI • H_2O.

6.2.7.1.2 Uses

Cysteine has many uses in industry not directly related to this discussion. In the food industry, cysteine is mainly used in artificial flavouring, especially the production of meat flavours (Huang and Ho, 2001). Cysteine is also used in the pharmaceutical and personal hygiene fields. Owing to cysteine's ability to break disulfide bonds in keratin, it is used to produce permanent hair waves (Starkenmann, 2008).

Cysteine's main medical application is as a supplement in total parenteral nutrition (TPN). Cysteine is naturally occurring in the body. In adults, it is synthesized from methionine via the trans-sulfuration pathway with the aid of the enzyme, cystathionase. Neonates have a relative deficiency of this enzyme, so cysteine hydrochloride may be indicated in neonates receiving TPN to ensure all amino acid requirements are met. (Drugs.com, 2012). Cysteine is metabolized to form pyruvate and inorganic sulfate. Cysteine can cause discolouration, and when added to TPN, it is advisable to check the bag for discolouration.

In Perfalgan® cysteine is added to protect paracetamol from oxidation. It is also postulated that the yellowish discolouration in Perfalgan® is due to cysteine.

6.2.7.1.3 NMR features

The ^{1}H NMR spectrum of cysteine hydrochloride analysis is displayed in Figure 3 below. The spectrum was recorded in deuterium oxide. It shows two multiplets. The one at 4.4 ppm is from the methylene proton while the peaks at 3.1 ppm are from the methane proton. The thiol and amine protons are not shown as they exchange with deuterium.

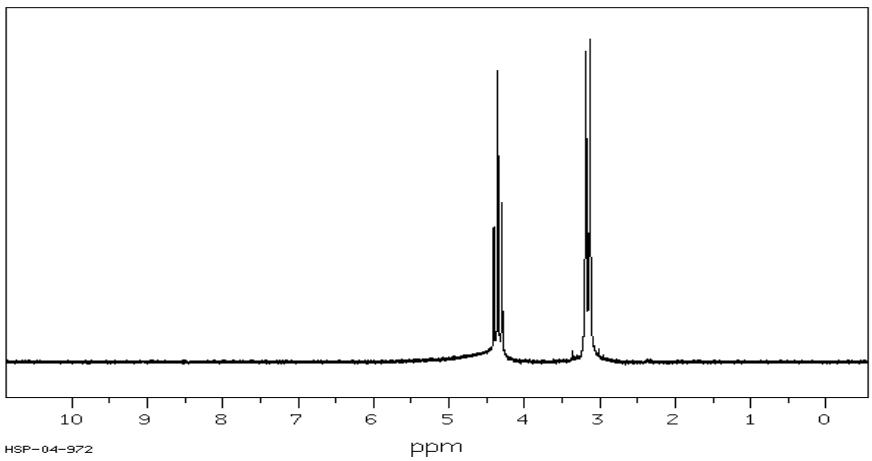


Figure 3: The NMR spectrum of cysteine hydrochloride

Figure 4 displays the NMR spectrum of oxidized cysteine (i.e. cystine). This spectrum is recorded in water and is far more complicated because the methylene protons have very different chemical shifts.

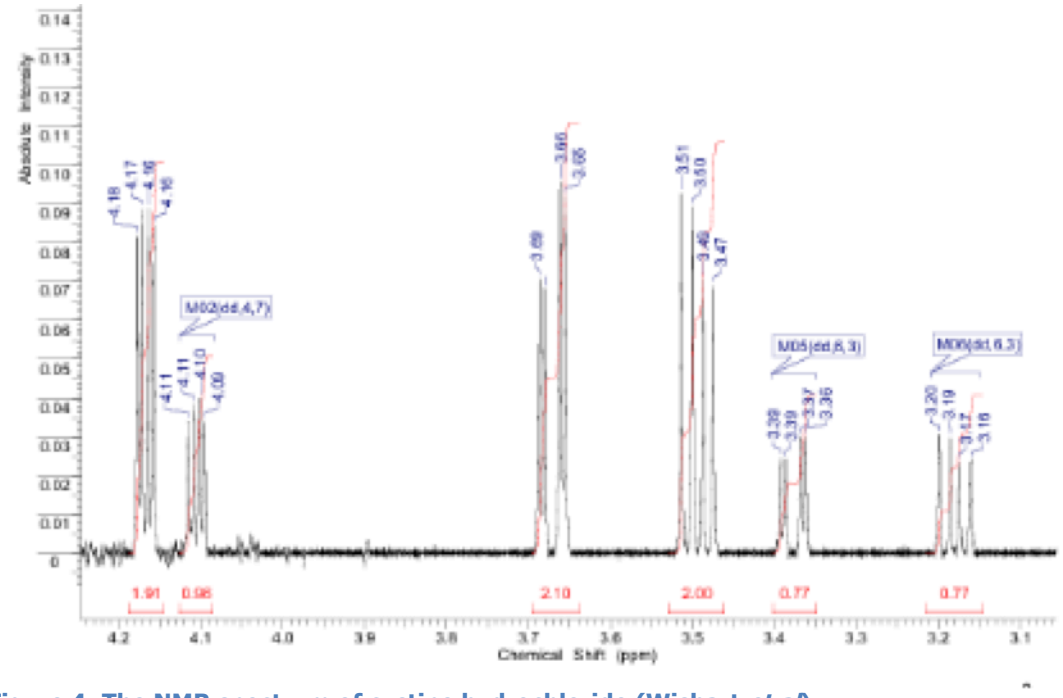


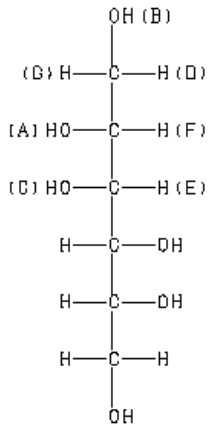
Figure 4: The NMR spectrum of cystine hydrochloride (Wishart *et al*)

6.2.7.2 Mannitol

6.2.7.2.1 Structure

D-mannitol is a polyol (sugar alcohol) with the chemical formula of $C_6 H_8(OH)_6$. It is available commercially in various white crystalline powder and granular forms, all of which are soluble in water (Shawkat, Westwood, & Mortimer, 2012).

Its structure is shown below.



6.2.7.2.2 Uses

Mannitol has several uses in the food and pharmaceutical industries. It is about half as sweet as sucrose and when taken orally has the desirable effect of masking bitter tastes. This makes it useful in the production of medicinal tablets (Shawkat, Westwood, & Mortimer, 2012).

In medicine mannitol is widely used in the management of raised intracranial pressure. When given intravenously it is filtered by the kidney because of its low molecular weight (182). It is not absorbed but remains osmotically active in the renal tubules, causing diuresis and naturesis (Nomani *et al*, 2014). It has also been used for renal protection in cardiac, vascular, and renal transplantation surgery, and in the management of rhabdomyolysis. Previously it has been used for bowel preparation before colorectal surgery (Shawkat, Westwood & Mortimer, 2012).

Mannitol is used in Perfalgan® as a solubilizing agent.

6.2.7.2.3 NMR features

The spectral output of D-mannitol analysis is displayed in Figure 5.

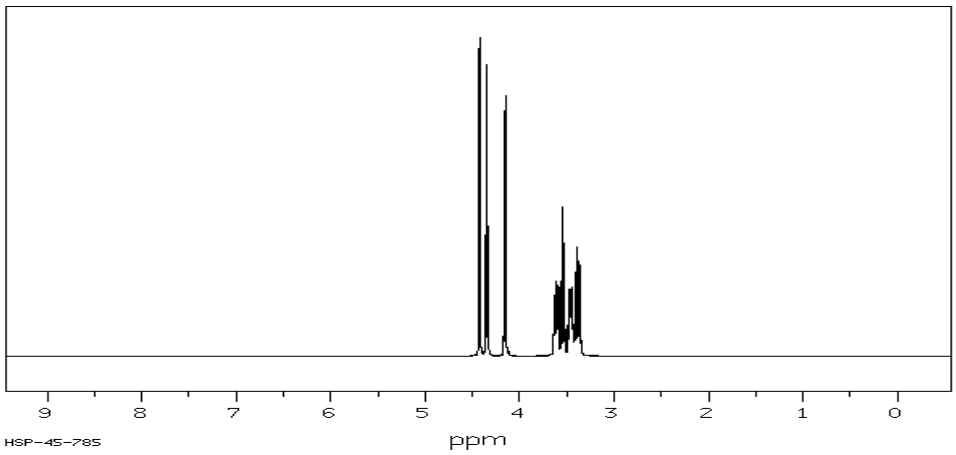


Figure 5: The NMR spectrum of D-mannitol

6.2.8 Use of Perfalgan® in the South African paediatric setting

The current practice of using one ampoule as a multidose vial represents a significant cost saving in the paediatric population. The cap of the vial is removed and a sterile hypodermic needle is used to puncture the rubber stopper and to withdraw the required dose into a syringe. The vial is kept on the anaesthetic trolley until the next case, when it is cleaned with an alcohol swab and the process repeated. At the end of the theatre day (usually less than 12 hours in duration), the vial is discarded.

This practice is employed as, at a recommended dosage of 15 $mg.kg^{-1}$, even the smaller sized 500 mg vial is more than is needed for many smaller children (<33kg). It is therefore cost-effective to reuse a vial on subsequent patients. It is not known however whether or not this practice results in underdosing of patients later in the day due to degradation of the active drug or alterations in excipients.

It is unknown if this practice is followed in other countries, especially where resource limitation is not as pertinent an issue.

6.2.9 Paracetamol stability testing

As previously mentioned, much work has been performed on the stability of oral paracetamol alone and in combination drugs. A PubMed search revealed limited research on intravenous paracetamol stability. Kwiatkowski, Johnson and Wagner (2012) tested samples of intravenous paracetamol with HPLC after exposure to air. 99% of the initial paracetamol concentration remained when tested over a period of 84 hours. Bernard *et al* (2012) examined intravenous paracetamol in infusion sets during the infusion and after 24 hours of stasis within the set. Chromographic analysis revealed paracetamol to be stable. The authors propose that successive doses of paracetamol may be infused through an administration set as the drug is stable.

No work was found focusing on the stability of intravenous paracetamol on exposure to heat or other stressors, or on the excipients in Perfalgan®.

7 RESEARCH QUESTION

Each 500mg or 1g vial is recommended for single use only and is to be discarded once opened (Medicines.org, n.d.). This is most likely due to concerns about degradation of paracetamol once exposed to air and sterility issues, although no evidence of these effects has been released by the manufacturers. However due to financial constraints, many centers in South Africa use one ampoule for multiple cases, especially in paediatric work. No obvious clinical adverse effects have been reported.

This study aims to answer the question, "Can Perfalgan® be used as a multidose vial or do the components degrade over time?" This will entail examining the stability of all components of Perfalgan® under various conditions, as detailed below.

8 PURPOSE OF THE STUDY

The purpose of this study is to measure the chemical and physical changes of Perfalgan® over time. The study will have 3 parts:

Part 1: Stability study of pure paracetamol and Perfalgan® over time.

Part 2: Stability study of additives over time, which may impact on solubility or bioavailability

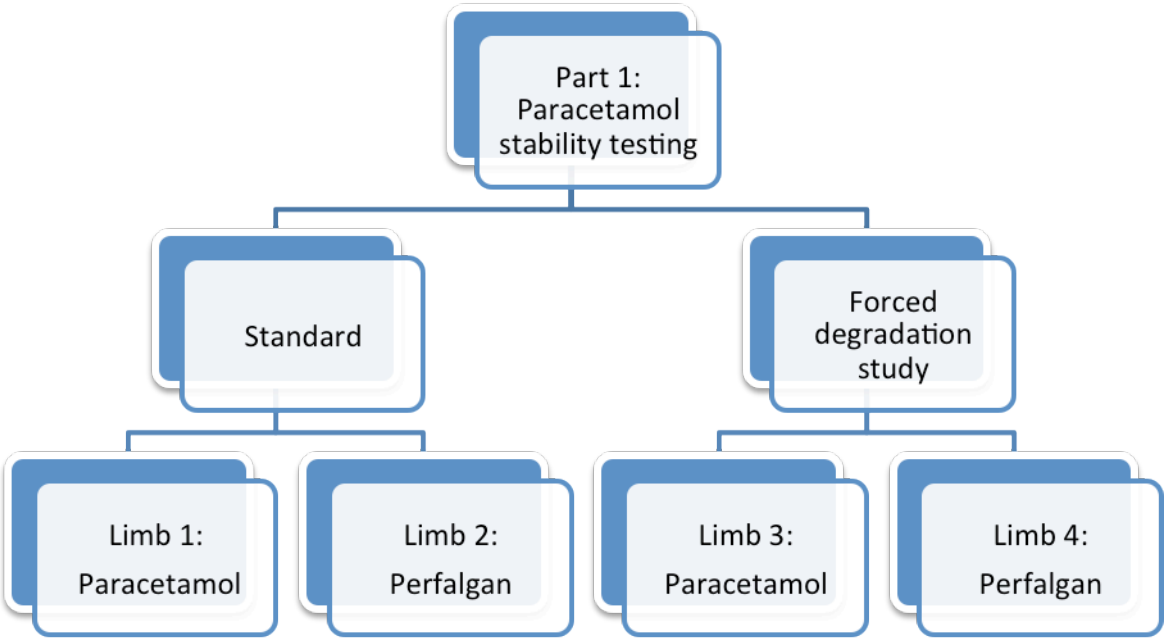
Part 3: Bioavailability study of Perfalgan® as indicated by penetration of paracetamol into a lipid layer.

9 METHODOLOGY

9.1 Part 1: Paracetamol stability study

Paracetamol stability was tested under 'unstressed' and 'stressed' conditions. The 'unstressed' sample was drawn from a vial that was exposed to air, simulating normal use. This is also termed standard conditions. 'Stressing' refers to placing the drug under conditions which may force it to degrade or alter its structure, namely by adding acid, alkali, an oxidizing agent and exposing to dry heat. This is known as a forced degradation study. The purpose of this exposure is to test, in the case of no degradation of paracetamol under 'unstressed' conditions, whether the methodology can detect change even under extreme conditions.

Part 1 of this study therefore has 4 limbs as depicted below. In limb one a stability study was performed on pure paracetamol on exposure to air at different time intervals. This served to calibrate the machinery and form a foundation for the other limbs of the study.



9.1.1 Equipment

As in experiments by Shah *et al* (2011), reverse phase high performance liquid chromatography with an ultraviolet detector (RP-HPLC)-UV was used to quantify paracetamol. In this process the analyte was dissolved within a liquid mobile phase, and injected into a column containing the stationary phase. Usually, one phase is hydrophilic and the other lipophilic. Depending on the polarity of the components in the analyte, they interact differently with the two phases. The more time spent interacting with the stationary phase, the more that component will be retarded, and vice versa. This delay leads to separation of the components of the sample. Each sample component elutes from the column at a specific time. This is termed its retention time (tR). Substances that are not retarded by the stationary phase leave the column at zero retention time, t0. The components are then passed through a detector which uses UV spectroscopy to record the signal as a chromatogram (Manz, Pamme and Lossifidis, 2005).

There are two modes of liquid chromatography in operation. In normal phase chromatography the stationary phase consists of a hydrophilic material (e.g. silica particles) and the mobile phase is a hydrophobic organic solvent (e.g. hexane). In reversed phase chromatography, the stationary phase is hydrophobic and the mobile phase is a mixture of polar solvents, (e.g. water and acetonitrile) (Manz, Pamme and Lossifidis, 2005). In this study, reverse phase high performance liquid chromatography (RP-HPLC) was used.

In this method, as described by Shah, *et al* (2011), a mobile phase of 0.05M potassium hydroxide phosphate:methanol (40:60 v/v) was used as it gives a sharp, well-resolved peak for paracetamol. The liquid chromatographic system of a SpectraSERIES 200 containing quaternary gradient pump, variable wavelength programmable UV/Vis detector and rheodyne injector with a 20 µl fixed loop was used. A 250 mm length and 4.6 mm internal diameter (i.d.) Thermo electron corporation C18 column with a 5 µm particle size was used in isocratic mode. A flow rate of 1 $ml.min^{-1}$ was used and the effluents monitored at 266 nm. The tR of paracetamol was expected to be approximately 2.7 minutes.

9.1.2 Equipment testing

9.1.2.1 Validation

Previous studies performed by Shah *et al*, showed the following parameters when analyzing paracetamol with a Brownlee C-18:

Table 3: Validation parameters of HPLC using Brownlee C-18. (Shah, *et al*., 2011)

Linearity range (µg.ml^{-1})	5-200
Slope	61190
Standard deviation of slope	47.69
Intercept	809.60
Standard deviation of intercept	298.04
Correlation coefficient	0.99
Detection limit (µg.ml^{-1})	0.54
Quantitation limit (µg.ml^{-1})	1.59
Accuracy (%)	98.24-100.07%
Precision(RSD,%) Intra-day precision (n=3) Inter-day precision (n=3)	 0.05-0.12% 0.07-1.45%
Instrument precision (RSD)	0.02%
Robustness	98.45-99.18%
Retention time (min)	2.7

The term RSD refers to the relative standard deviation. The RSD value for paracetamol is 0.02%. This is calculated by performing injection repeatability tests as explained below. The low RSD value indicates that the method is precise. The high value of 98.24-100.07% indicates the high accuracy of this method.

The detection limits for paracetamol (i.e. the lowest concentration at which paracetamol can be reliably differentiated from background levels) is 0.54 µg.ml^{-1}. The quantitation limit (i.e. the

lowest concentration at which the drug can be quantified) was 1.59 µg.ml^{-1}. Both these limits show that the quantity of the drug can be accurately and precisely determined.

The R^2 value is the goodness-of-fit statistical method used in this study. In linear regression analysis it is the square of the correlation coefficient referred to by Shah *et al*. It measures how successfully the regression line expresses the variance of the data. It is a value between 0 and 1 (Kolobe, 2006). An R^2 value of greater than 0,95 means that there is 95% confidence that the equation fits the data.

9.1.3 Procedure

9.1.3.1 Preparation of mobile phase and paracetamol stock solution

The mobile phase solution was prepared by accurately weighing 6.8 g of potassium dihydrogen phosphate and dissolving it in 1000 ml of water. 800 ml of this solution was mixed with 1200 ml of methanol and filtered with Anatech Instruments Ltd. nylon filter paper (0.45 µm). The solution was sonicated with a Julaba USR3 for 15 minutes for degassing prior to use.

The paracetamol stock solutions were made at varying concentrations in order to calibrate the HPLC machine. 1.3 mg, 2.61 mg, 5.13 mg and 7.53 mg of paracetamol were accurately weighed then each added to a 40:60 mixture of boiled, distilled water and methanol (4 ml water and 6 ml methanol). This resulted in 0.13 mg.ml^{-1}, 0.261 mg.ml^{-1}, 0.513 mg.ml^{-1} and 0.753 mg.ml^{-1} paracetamol solutions respectively. Each solution was filtered using a NY 0.22 µm filter to ensure no undissolved particles remained in the sample. 1.5 ml aliquots of each paracetamol solution were injected into the HPLC sample vials.

9.1.3.2 Preparation of Perfalgan® solution

Perfalgan® stock solution was prepared by accurately drawing 0.5 ml of Perfalgan® and adding to 10 ml of mobile phase resulting in a total volume of 10.5 ml. Since it is already a liquid there was no need to filter this solution. The final concentration of paracetamol was therefore 0.476 mg.ml^{-1}.

9.1.3.3 Storage of solutions

The stock solutions were stored on the laboratory bench between sampling. The laboratory temperature was regulated at 20°C.

9.1.3.4 Calibration

In preliminary investigations aliquots of paracetamol standard solution at different concentrations as described above were used. These solutions were injected using a 20 µL fixed loop system and chromatograms recorded. Calibration curves were constructed by plotting peak area vs. concentration of the drug.

9.1.3.5 Measurement of paracetamol

A fixed volume of 20 µL of the solution was injected using a fixed loop into the HPLC-UV machine and the tR determined. By integration of the area beneath the peak, the concentration of paracetamol was obtained. This was repeated at various concentrations of paracetamol to ensure linearity and accuracy of the machine. A R^2 value of better than 0.95 was regarded as acceptable.

9.1.4 Tests performed

9.1.4.1 Limb one: Paracetamol under standard conditions

This was performed by testing pure paracetamol stability and calibrating the equipment.

9.1.4.2 Limb two: Perfalgan® under standard conditions

This consisted of repeating the paracetamol assay procedure using Perfalgan® instead of pure paracetamol. The paracetamol peak was examined in a sample of stock solution at 0 hr and 24 hr.

9.1.4.3 Limb three: Paracetamol forced degradation study

According to Shah *et al* (2011) paracetamol does not degrade when exposed to acid, base, heat and oxidative stress.

In this study, a forced degradation study was carried out by subjecting paracetamol stock solution to acid and alkali hydrolysis, chemical oxidation and dry heat. This was achieved by adding an acid or base, or in the case of dry heating, by heating the sample to 80°C for 30 minutes.

9.1.4.3.1 Basic solution

A 1 N NaOH solution was prepared by mixing 5.2 ml of 52% NaOH and 94.8 ml water (ie total volume 10ml). 2.5ml of Perfalgan® stock solution was placed in a volumetric flask and 5 ml of 1 N NaOH was added. The solution was neutralized with acid using indicator paper. Mobile phase was added up to the 25 ml mark to give a final concentration of 47.6 $\mu g.ml^{-1}$ of paracetamol.

9.1.4.3.2 Acidic solution

The above method was adjusted only by adding 1 N HCl. The 1 N HCl solution was prepared by diluting 9.8 ml of 32% HCl in 9.2 ml water (ie total volume 10 ml).

9.1.4.3.3 Oxidative stress solution

Hydrogen peroxide was prepared by diluting 30% hydrogen peroxide tenfold (i.e. 3% H_20_2). 2.5 ml of stock solution was placed in a 25 ml volumetric flask and 5 ml of 3% H_20_2 was added. Mobile phase was added up to the 25 ml mark.

9.1.4.3.4 Heat degradation

To study dry heat degradation, solid paracetamol was exposed in an oven at 80°C for 30 minutes. Thereafter 25 mg of paracetamol was transferred into a 25 ml volumetric flask and diluted up to the 25 ml mark with the mobile phase. The solution was further diluted to 5 $\mu g.ml^{-1}$ for analysis.

9.1.4.4 Limb four: Perfalgan® forced degradation study

Once the forced degradation study had been performed on paracetamol, it was repeated with Perfalgan®. The samples were inspected for discoloration and crystal formation. The concentration of paracetamol and its oxidation products in each sample was compared to determine if significant degradation had occurred in response to those stressors.

9.1.5 Statistical analysis of experimental method

9.1.5.1 Calculation of an unknown concentration from the calibration curve

Where a calibration curve yields a straight line, the formula can be expressed as y=mx + c (where 'm' represents the slope and 'c' the y intercept). It follows that:

$x = \frac{y - c}{m}$ therefore

the unknown concentration = $\frac{reading\text{-}intercept}{slope}$

9.1.5.2 Calculation of standard error

In order to calculate the error in the unknown concentration when using the calibration curve, account had to be taken of the error of the calibration curve itself. The standard error in the unknown concentration was calculated using the following equation:

$$s_{x_0} = \frac{s(r)}{m}\sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(\bar{y}_o - \bar{y})^2}{m^2 \sum_{i=1}^{n}(x_i - \bar{x})^2}}$$

Where:

$s(r)$	is the residual standard deviation
n	is the number of paired calibration points (x_i, y_i)
m	is the calculated best-fit gradient of the calibration curve
N	is the number of repeat measurements made on the sample (this can vary from sample to sample and can equal 1)
$\bar{y}_o$	is the mean of N repeat measurements of y for the sample
$\bar{y}$	is the mean of the y values for the calibration standards
x_i	is a value on the x-axis
$\bar{x}$	is the mean of the x_i values

A confidence interval was obtained by multiplying s_{x0} by the 2-tailed Student t value for the appropriate level of confidence and n-2 degrees of freedom (Barwick, 2003).

9.2 Part 2: Study of excipient degradation in Perfalgan®

The second component of study used ^{1}H NMR (nuclear magnetic resonance). This technique works on the principle that each proton in different compounds resonates at a different and characteristic frequency. The pattern of the specific peaks within the substance can be used to describe the compound and its degradation products. This is the main analytical tool used in organic chemistry to identify unknown compounds. In this way the changes in the concentration of cysteine and mannitol in Perfalgan® over time were studied.

9.2.1 Equipment

A Bruker 400 ^{1}H NMR spectrometer with a BBI inverse detection probe was used. Excitation sculpting was used to suppress the water signal, as explained below.

^{1}H NMR is based on the principle of proton motion within the nucleus of a compound. The proton nucleus of a compound has a spin quantum number of ½. This means that it can spin clockwise

or anticlockwise. These two spins have the same energy. However, when a proton is placed in a strong magnetic field the two spins have different energy. In NMR the difference in energy between these two spin states is measured. The difference in energy is dependent on the strength of the magnetic field that the nucleus experiences. Since different protons in a given sample are in different chemical environments they will be shielded differently from the strong magnetic field that the sample is exposed to. Therefore the energy or frequency of the signal is a measure of the chemical environment of the proton. Since most chemical compounds consist of many different protons, a different signal can be detected from each different proton. These signals are used to identify the compound. In essence, NMR can be compared to 'fingerprinting' a compound. The output of this machine is a ^{1}H spectrum. By comparison with standard tables it is possible to match the spectrum with a compound.

If unable to identify the compound, the ^{13}C signal and Heteronuclear Single Quantum Coherence (HSQC) can be measured. Both these tests are lengthy.

Normally NMR samples are dissolved in deuterated solvents so that the solvent signals do not appear in the spectrum. (The sample concentration is in the mmolar range and water is 55.5 M.). Since Perfalgan® already contains water the methodology of this study needed to be adjusted. Deuterium could not be used because the sample is already dissolved in water not deuterium oxide (D_2O). Instead specific pulse sequences were used to suppress the water signal. This is known as excitation sculpting.

A ^{1}H NMR spectrometer requires no calibration.

9.2.2 Sample preparation

The Perfalgan® stock solutions and those exposed to acid, base, heat and oxidative stress prepared in the Part 1 of the study were used. 500 µl aliquots were mixed with 100 µl of D_2O and placed in a NMR sample tube.

9.2.3 Tests performed

Each sample was analysed with ^{1}H NMR. The Bruker was locked on the deuterium signal. Each run took approximately 10 minutes. Where change in the sample was suspected, it was subjected to further testing using HSQC and ^{13}C analysis. These were run overnight.

9.3 Perfalgan® lipid penetration study using octanol:water coefficient

Paracetamol is believed to have predominantly central activity. Penetration into a lipid layer is therefore necessary for the effect to take place. In order to assess this penetration the aqueous-octanol partition coefficient for Perfalgan® was performed. Octanol is an organic solvent frequently used for this purpose in pharmaceutical testing. According to the literature, the octanol:water partition coefficient ($logP_{ow}$) of paracetamol is 0.46 — 0.49 (International Programme on Chemical Safety, 2008; McNeil Consumer Healthcare, 2010).

A partition coefficient is the ratio of concentrations of a compound in a mixture of two immiscible phases at equilibrium. One solvent is usually hydrophilic (or water itself) and one hydrophobic, such as octanol. The octanol:water partition coefficient is therefore a measure of the difference in solubility of the compound in these two phases (Leo, Hansch and Elkins, 1971).

This coefficient is useful for predicting the distribution of drugs in the body. Hydrophobic drugs have a high octanol:water partition coefficient and therefore preferentially distribute to hydrophobic compartments, such as the brain and fat. Hydrophilic drugs conversely have a low octanol:water partition coefficient and distribute mainly in hydrophilic compartments, such as the blood.

The octanol: water partition coefficient is calculated using the following equation:

$$\log\ P_{\mathrm{oct/wat}} = \log\left(\frac{[\mathrm{solute}]_{\mathrm{octanol}}}{[\mathrm{solute}]^{\mathrm{un-ionized}}_{\mathrm{water}}}\right)$$

In this study octanol was added to the paracetamol and Perfalgan® stock solution and UV spectroscopy performed on the aqueous and lipid (or organic) layers to assess the concentration of paracetamol in each. From there the octanol:water partition coefficient was determined.

9.3.1 Paracetamol calibration

1 mg of paracetamol was accurately weighed out and dissolved in 10 ml of 40:60 water:methanol. 100 μl of this solution was diluted in a further 10 ml of 40:60 water:methanol resulting in a 1 $\mu g.ml^{-1}$ solution. Similarly 2, 3, 4, 5 and 6 $\mu g.ml^{-1}$ solutions were mixed. 2 ml of each solution was added to 2 ml of octanol and shaken for 30 seconds before leaving to stand for 10 minutes. The mixture separated into two layers, with the octanol or organic layer lying on top of the aqueous layer. The octanol layer was analysed with UV spectroscopy and a calibration curve for lipid penetration of paracetamol drawn. The aqueous layer was also analysed so the octanol:water partition coefficient is obtained.

9.3.2 Perfalgan® testing

Perfalgan® stock solution was similarly diluted to a paracetamol concentration of 5 $\mu g.ml^{-1}$. 2 ml of this solution was added to 2 ml of octanol and the procedure repeated as for paracetamol above. Similarly Perfalgan® samples were exposed to heat, acid, base and peroxide as in Part 1. UV spectroscopy was used to measure the absorbance of paracetamol in each layer of the 'stressed' and 'unstressed' samples. The calibration curve was used to calculate the concentration of paracetamol in the octanol layer of each sample. The octanol:water partition coefficients were also calculated and tabulated. A change in the concentration of paracetamol in the organic layer or the

partition coefficient, may suggest that the bioavailability of the paracetamol in Perfalgan® was affected.

9.4 Repetition of experiments

This study made use of three different analysis techniques, namely HPLC, ^{1}H NMR and UV spectroscopy. Preliminary HPLC assays were repeated to establish ideal experimental conditions. However, since the study was not quantitative and time on the instrument was limited, multiple runs of the final samples were not performed. ^{1}H NMR was performed on several samples and at different times. Where quantitative analysis was required, ie when testing the log P_{ow} of Perfalgan® with UV spectroscopy, multiple runs were performed to enable accurate calculation of statistics.

10 RESULTS

10.1 HPLC results

Initial work was done to confirm that chromatograms of pure paracetamol and the paracetamol in Perfalgan® were the same. The Perfalgan® samples were tested at 0 hours, ie the rubber stopper was pierced and the first sample drawn was immediately analysed. Figures 6 and 7 show the chromatograms generated.

10.1.1 Identification of paracetamol in Perfalgan®

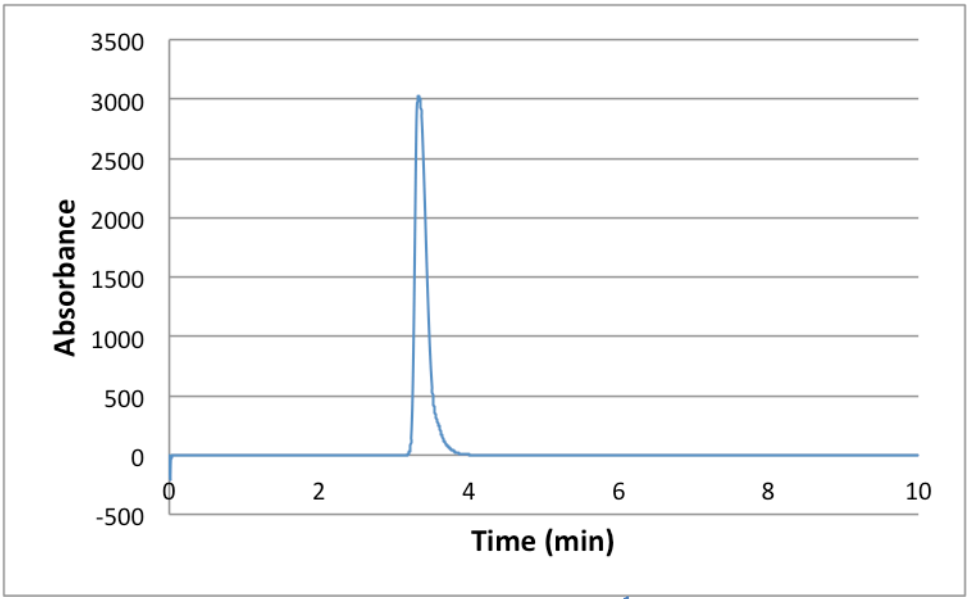


Figure 6: Chromatogram of paracetamol 513 µg.ml^{-1} at 0 hours

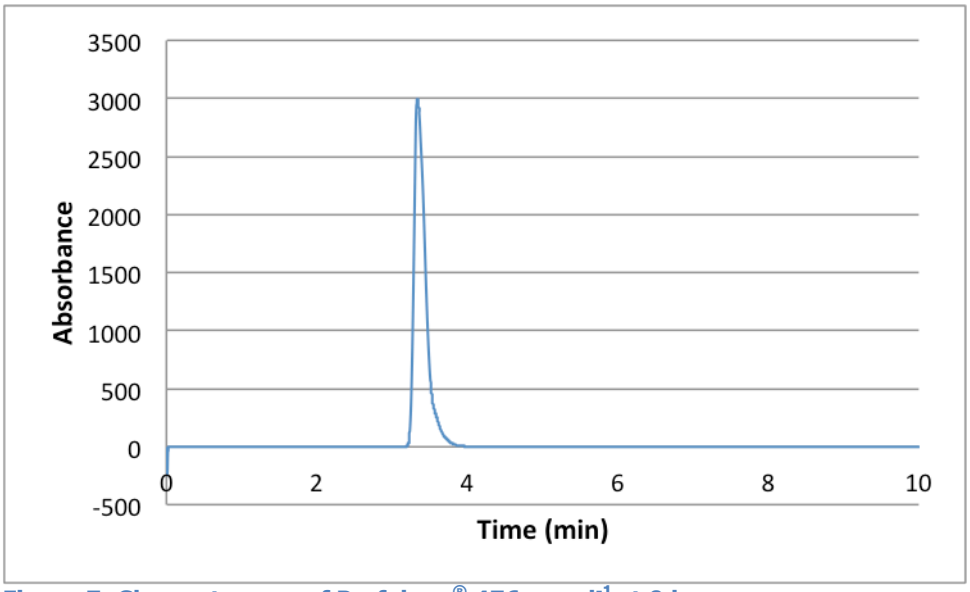


Figure 7: Chromatogram of Perfalgan® 476 µg.ml^{-1} at 0 hours

The similarities of the tR identified the paracetamol in the Perfalgan® within the limits of experimental error.

10.1.2 Quantifying paracetamol in Perfalgan®

The calibration curve for paracetamol is displayed in figure 8. The absorbance was measured at 266 nm between the concentrations of 100 and 800 µg.ml^{-1}. These data yielded a straight line with the equation of y= 47524x + 8X10^{6}. The R^2 value was 0.98. This was deemed an acceptable trend line because the R^2 was greater than 0.95 meaning it is above the 95% confidence interval.

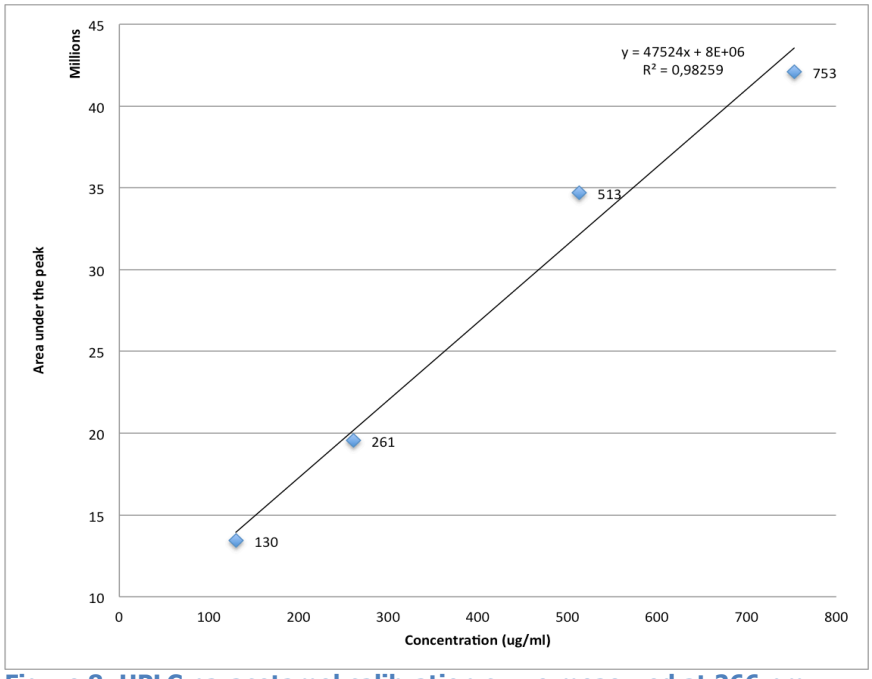


Figure 8: HPLC paracetamol calibration curve measured at 266 nm

The area under the peak (in million units) is correlated to varying concentrations of paracetamol. The equation of the straight line is displayed where 'E' represents 'exponent' (ie 8E+06 = 8X10^6).

Table 4: HPLC paracetamol calibration curve parameters

Correlation coefficient	0.98
Standard error	2.18
Slope	48000 μg^{-1}
Standard deviation in slope	5000
Intercept	7.76 x 10^6
Standard deviation in intercept	2.17 x 10^6

10.1.3 The effect of changing environment on Perfalgan®

Chromatograms were generated for paracetamol and Perfalgan® under stressed conditions as per limb 3 and 4 of the study. The chromatograms of paracetamol remained unchanged after exposure to heat, acid, base and H_2O_2. This was in agreement with extensive literature on paracetamol stability and therefore only the chromatograms of Perfalgan® were included in the sections below.

10.1.3.1 Exposure to air

The chromatogram of Perfalgan® on exposure to air shows no degradation of paracetamol after 24 hours (cf Figure 9).

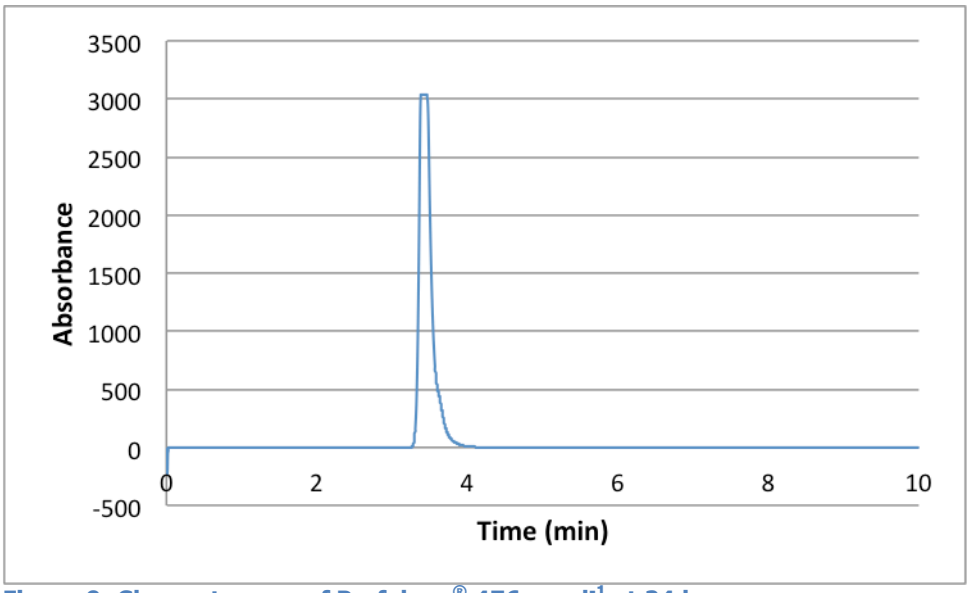


Figure 9: Chromatogram of Perfalgan® 476µg.ml^{-1} at 24 hours

The paracetamol calibration curve was used to calculate the concentration of paracetamol in each sample of Perfalgan®.

Table 5: Comparison between the area under the peak measured by HPLC (in million units) and the paracetamol concentration (in µg.ml^{-1}) in Perfalgan® at 0 and 24 hours

	Standard	
	0 hr	24 hr
Area under curve	32,4	36.4
Concentration	520	600
Standard error	50	50
95% Confidence interval	220	230

The standard errors are quite high due to the standard deviation of the slope. This could have been improved by using more calibration points and or replicates.

Summary:

As depicted in Table 5, the measured concentration of paracetamol was 0.5 mg/mL, which is the manufacturer's reported concentration in the vial. Within the experimental error there was no loss of paracetamol over 24 hours.

10.1.3.2 Exposure to acid

The representative chromatograms of 0 hours and 24 hours are shown in figures 10 and 11 below.

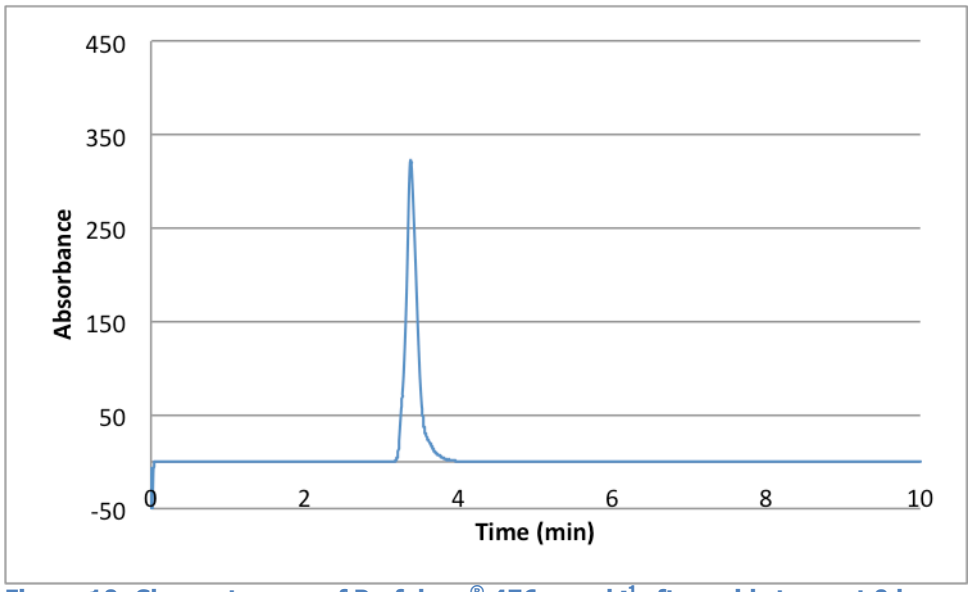


Figure 10: Chromatogram of Perfalgan® 476 µg.ml $^{-1}$ after acid stress at 0 hours

(Analysis at 0 hours means that the added acid was neutralized with base immediately, then the sample was tested.)

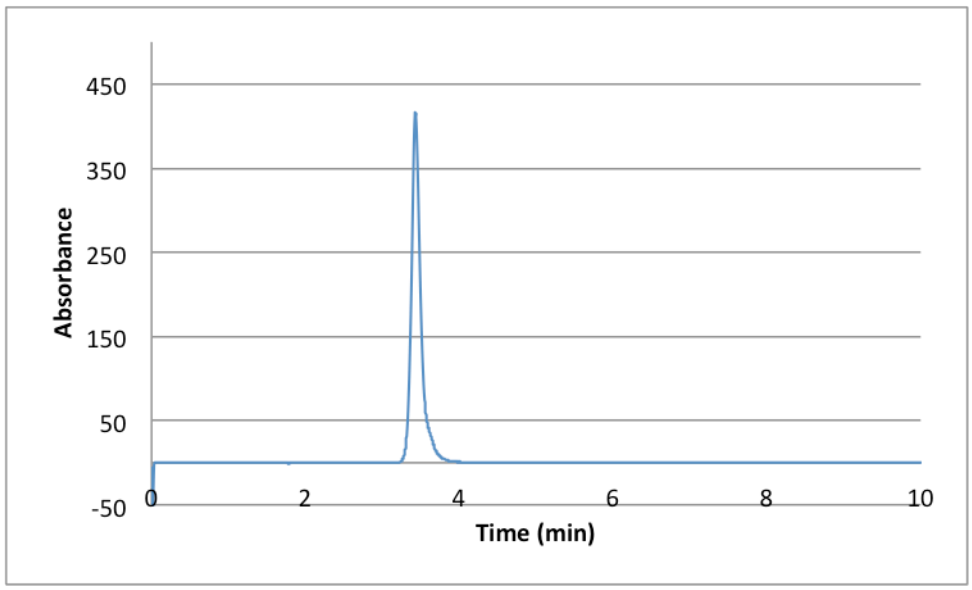


Figure 11: Chromatogram of Perfalgan® 476 µg.ml $^{-1}$ after 24 hours of acid stress

(*Analysis at 24 hours means that acid was neutralized with base 24 hours after initial exposure, then the sample was tested.)*

Summary:

On exposure to acid there was visible precipitation within the Perfalgan® sample. The HPLC results confirmed this with < 10 % of the paracetamol remaining in solution. Although the HPLC results showed a decrease in the concentration they also showed the same retention time and that no degradation peaks were present. The same chromatogram was obtained after 24 hours. Due to the precipitation, the calibration curve was not applied as concentrations calculated would be meaningless.

10.1.3.3 Exposure to base

Mixing the standard solution of Perfalgan® with base resulted in copious precipitation. While the supernatant could have been analysed by HPLC, judging by the amount of precipitate it was concluded that all the paracetamol had precipitated. Given this assumption and the danger of using high concentrations of base on the HPLC column it was decided not to proceed further with this experiment.

10.1.3.4 Exposure to hydrogen peroxide

The representative chromatograms of 0 hours and 24 hours are shown in Figures 12 and 13 below.

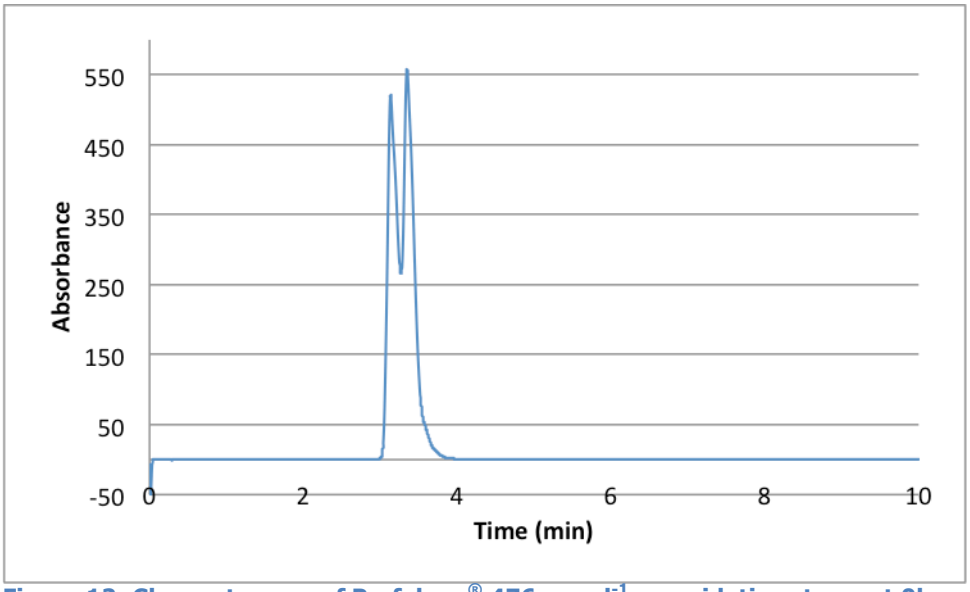


Figure 12: Chromatogram of Perfalgan® 476 µg.ml^{-1} on oxidative stress at 0hr

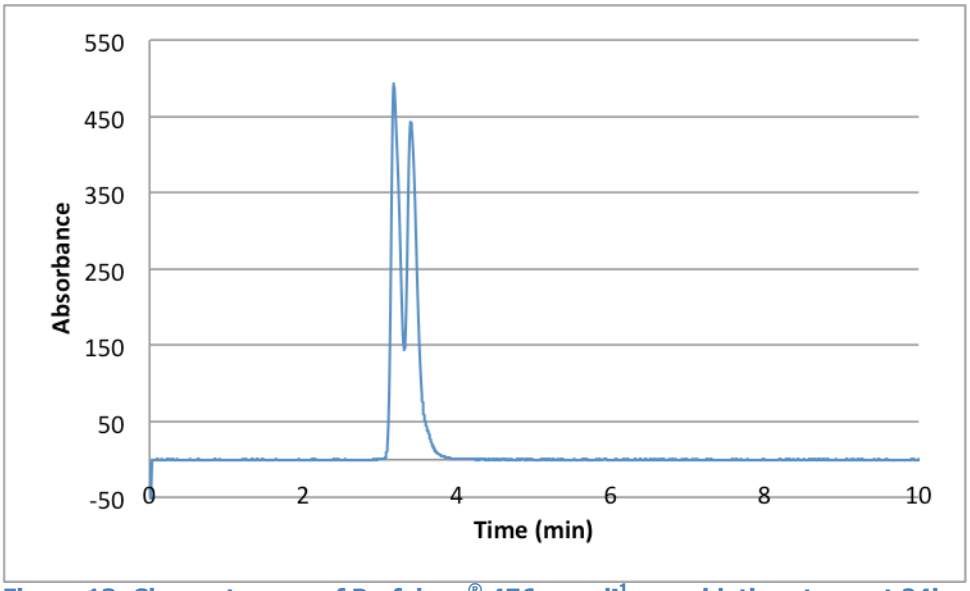


Figure 13: Chromatogram of Perfalgan® 476 µg.ml^{-1} on oxidative stress at 24hr

Summary:

Hydrogen peroxide caused splitting of the paracetamol peak. The reason for this is discussed later.

10.1.3.5 Exposure to dry heat

The representative chromatograms of 0 hours and 24 hours are shown in Figures 14 and 15 below.

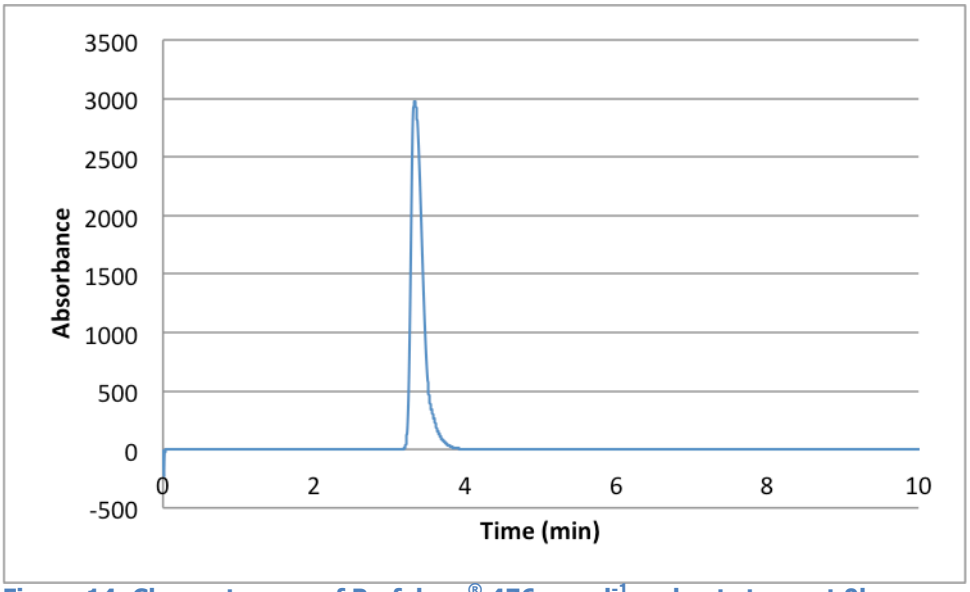


Figure 14: Chromatogram of Perfalgan® 476 µg.ml^{-1} on heat stress at 0hr

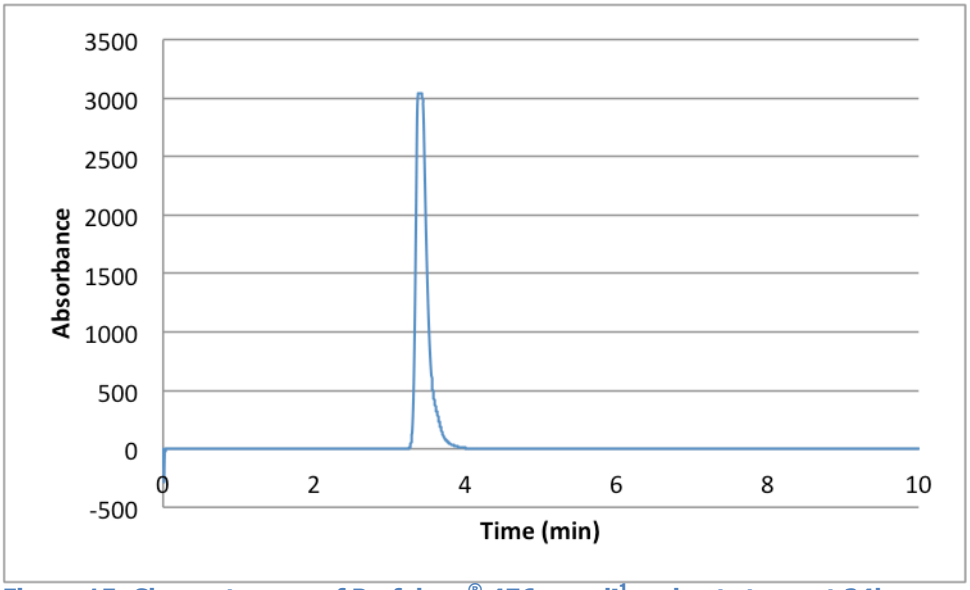


Figure 15: Chromatogram of Perfalgan® 476 µg.ml^{-1} on heat stress at 24hr

Table 5: Comparison between the area under the peak measured by HPLC (in million units) and the paracetamol concentration (in µg.ml^{-1}) in Perfalgan® on exposure to dry heat

	Heat Stressed	
	0 hr	24 hr
Area under curve	33.14	33.05
Concentration	502,7	501,1
Standard error	50	50
95% Confidence interval	230	230

Summary:

The paracetamol in Perfalgan® did not degrade on heat exposure.

10.1.3.6 Summary of all HPLC findings

Quantitative analysis of the paracetamol in Perfalgan® proved difficult using HPLC due to precipitation in the acid- and base-stressed samples and splitting of the peak as in the case of H_2O_2 exposure. However, quantitative results from the unstressed and heat stressed samples confirmed the of paracetamol in vial to conform to the manufacturer's specification. No loss was seen with time. Qualitative analysis showed that samples had the same tR and no degradation products of paracetamol were seen.

10.2 ^{1}H NMR results

^{1}H NMR was performed on Perfalgan® only as it was the method used to specifically study the excipients in Perfalgan®. Samples were studied under the 'stressed' and 'unstressed' conditions used previously, creating the following spectra. The x-axis shows the proton chemical shift which is a measure of the magnetic shielding of the nucleus and hence its chemical environment. The y-axis shows the absorption intensity. The area under the peak is proportional to the number of protons in the compound.

10.2.1 Unstressed Perfalgan® spectra

10.2.1.1 Perfalgan® at 0hr

Standard tables (NMRShiftDB, 2008) were used to identify the main constituents in Perfalgan®, namely paracetamol, cysteine and mannitol. In Figures 16-20, the cysteine spectrum is shown as an insert at two hundred times magnification as its concentration is much lower than the other two constituents.

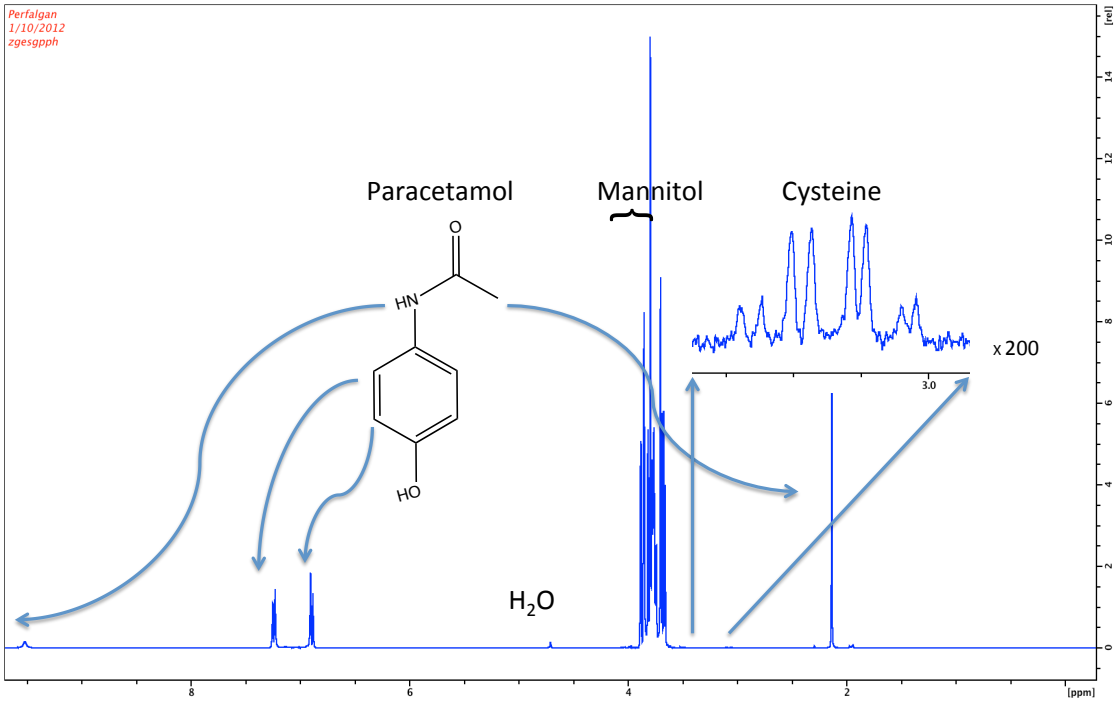


Figure 16: ^{1}H NMR spectrum of Perfalgan®

The peaks from cysteine and the protons on the benzene ring of paracetamol show 'fine structure' from spin-spin coupling, which helps identify these compounds. Mannitol has a complex spectrum, typical of sugars. Some impurities were seen at ~2 and 3 ppm in the sample, at a concentration similar to the cysteine.

Using integration of the ^{1}H NMR data, it was possible to calculate the concentration of each constituent in Perfalgan® as the concentration of paracetamol is known to be 10 mg.ml^{-1}.

Table 6: Comparison of the major three constituents in Perfalgan®

Constituent	Molar ratio derived by ^{1}H NMR	Molecular weight ($g.mol^{-1}$)	Concentration ($mg.ml^{-1}$)
Paracetamol	1	151,17	10
Mannitol	1,24	182,17	14,97
Cysteine	0,004	121,16	0,03

Since cysteine is at a much lower concentration than either paracetamol or mannitol, the cysteine region has been displayed magnified in an insert in Figures 15-18. Where two time periods are shown on the same figure, the spectra are offset slightly for clarity (cf: Figures 17 and 18).

10.2.1.2 Perfalgan® at 48hr

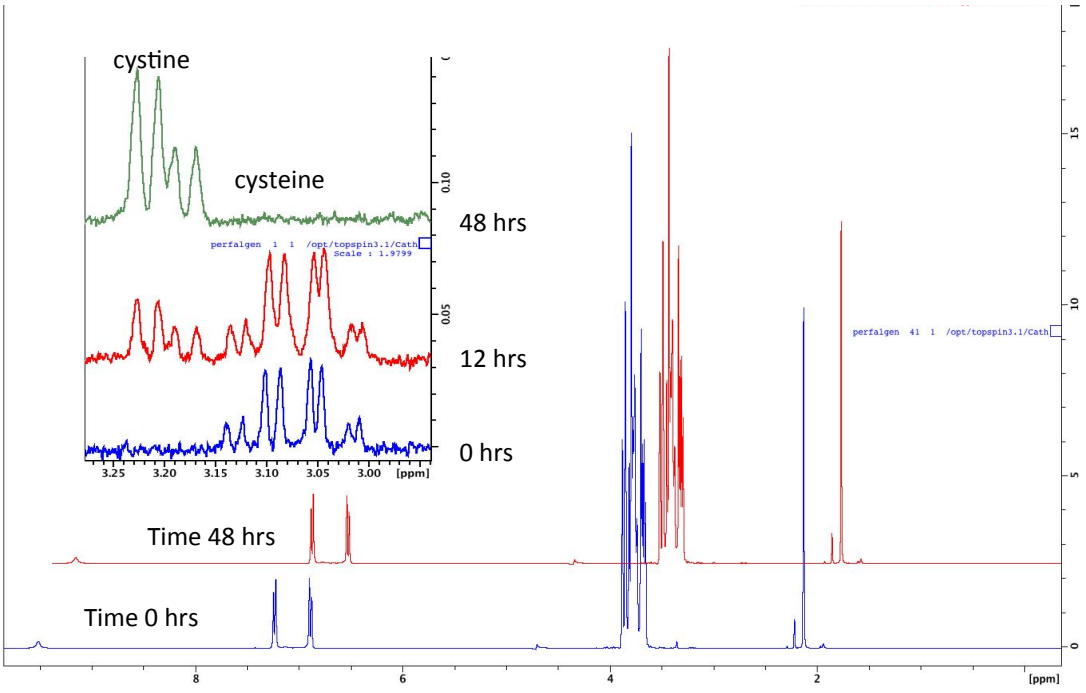


Figure 17: ^{1}H NMR spectrum of Perfalgan® at 0 hr and after 48 hr of exposure to air

Summary:

On exposure to air, over time, cysteine oxidised to form cystine. There was no change in the paracetamol or mannitol.

10.2.2 The effect of changed environment

10.2.2.1 Acid stress

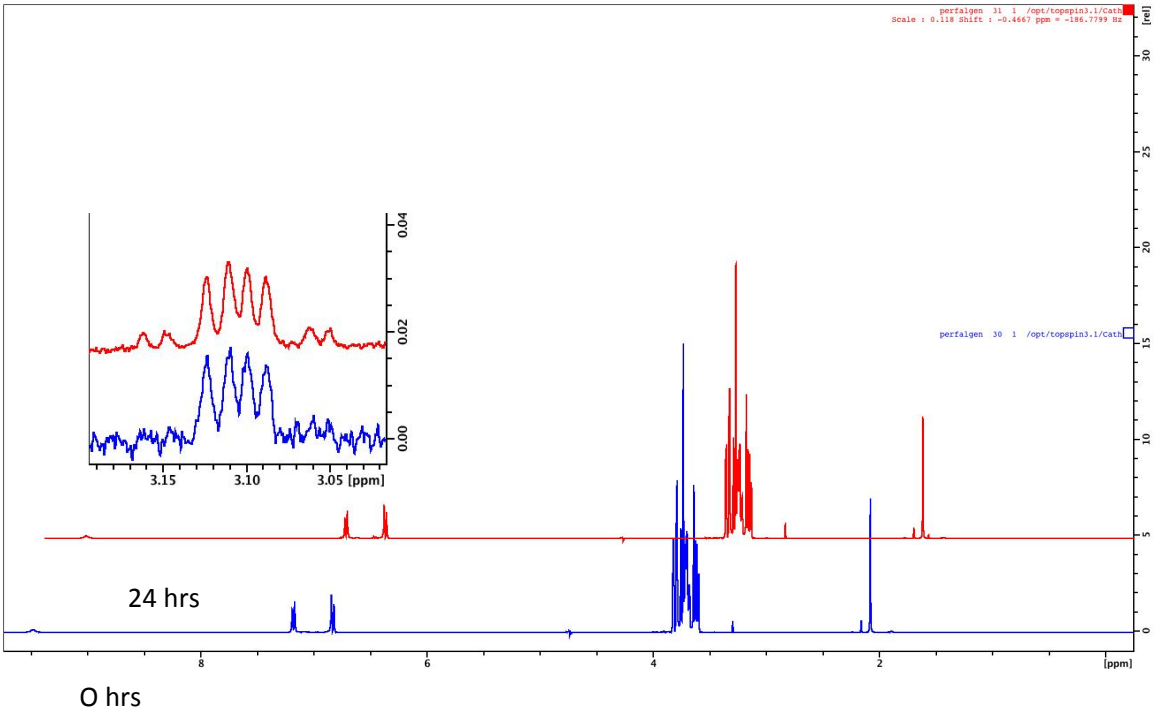


Figure 18: ^{1}H NMR of Perfalgan® showing the effect of acid

Summary:

The cysteine insert showed some chemical shift changes because of the change in pH but otherwise the spectrum was the same as pure Perfalgan®. Again there was no change in the paracetamol and mannitol.

10.2.2.2 Heat

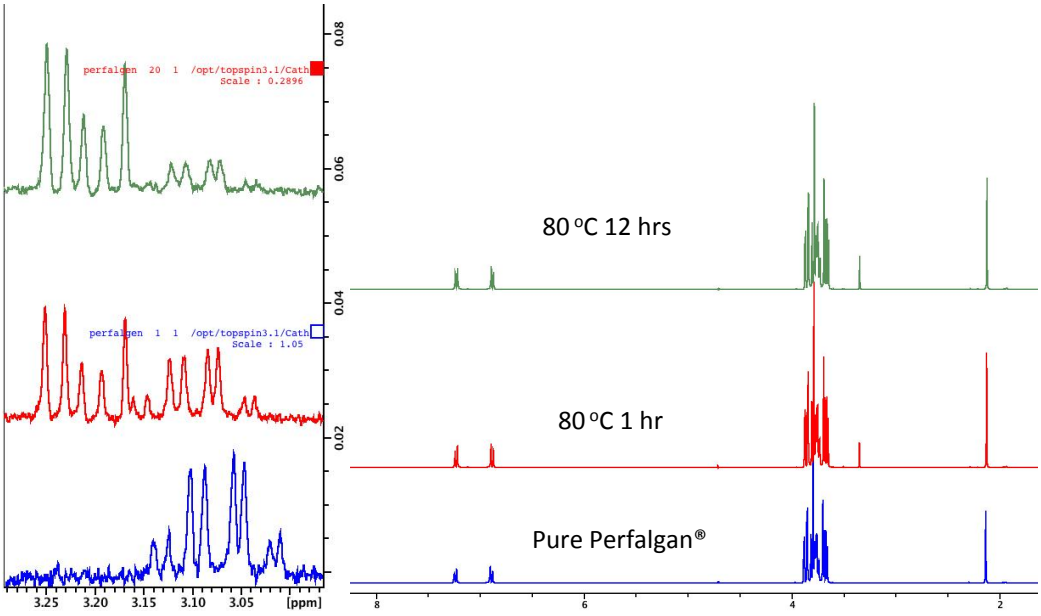


Figure 19: ^{1}H NMR spectrum of Perfalgan® showing the effect of heat

Summary:

When heated there was no change in paracetamol or mannitol in Perfalgan®. Again, the cysteine oxidised to form cystine as air was not excluded from the experiment. This change occured more quickly under the influence of heat. All cysteine was converted to cystine within 12 hours, as opposed to 48 hours in the unstressed sample.

10.2.2.3 Hydrogen peroxide stress

10.2.2.3.1 ^{1}H NMR

In some cases, the hydrogen peroxide sample developed a yellow colour after 24 hours. However, no difference was noted in the ^{1}H NMR, HPLC nor UV spectroscopy of these samples.

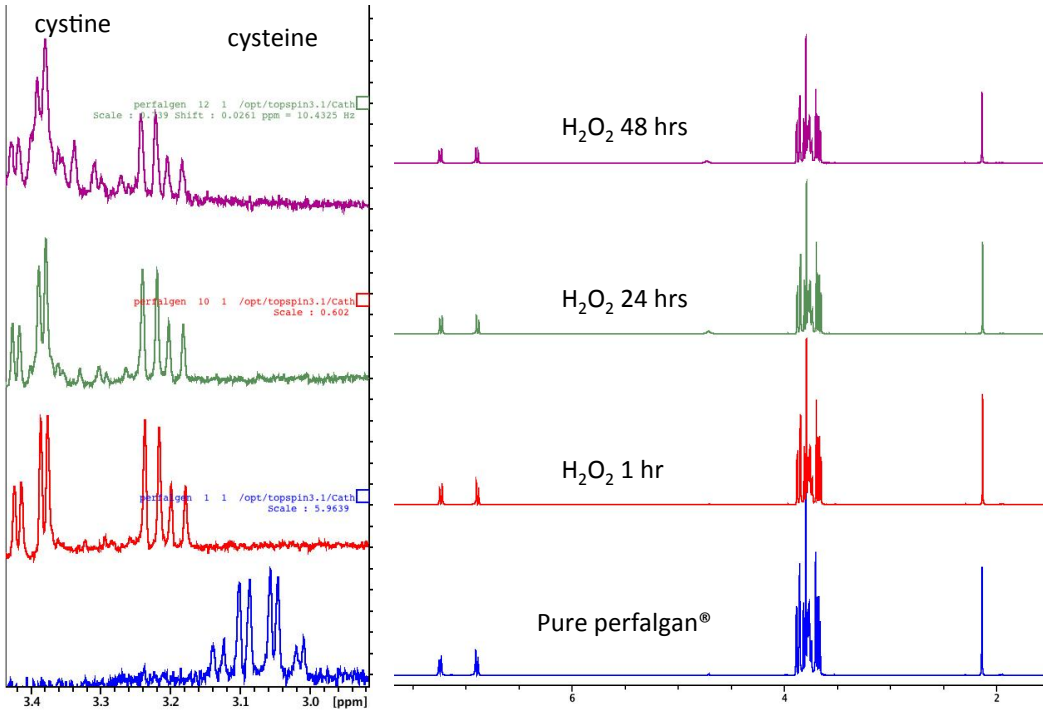


Figure 20: ^{1}H NMR spectrum showing the effect of H_2O_2

Under hydrogen peroxide stress again the paracetamol and mannitol did not alter. The cysteine converted to cystine rapidly and completely within 1 hour.

In order to further analyse the yellow discolouration on exposure to hydrogen peroxide, the HSQC and ^{13}C spectrum were recorded to see if a new compound had been formed. (see below)

10.2.2.3.2 HSQC

HSQC is an indirect correlation experiment in which protons are correlated to directly bonded carbons. It enables the ready assignment of the other resonance once one is known. In the 2D spectrum, the x-axis shows the chemical shift of the ^{1}H proton and the y-axis, the shift of the carbon. The cross-peaks in the centre of the plot correlate the two chemical shifts via scalar coupling. All the proton peaks correlated with their respective ^{13}C peaks. The impurities and cysteine were at too low a concentration to show correlations.

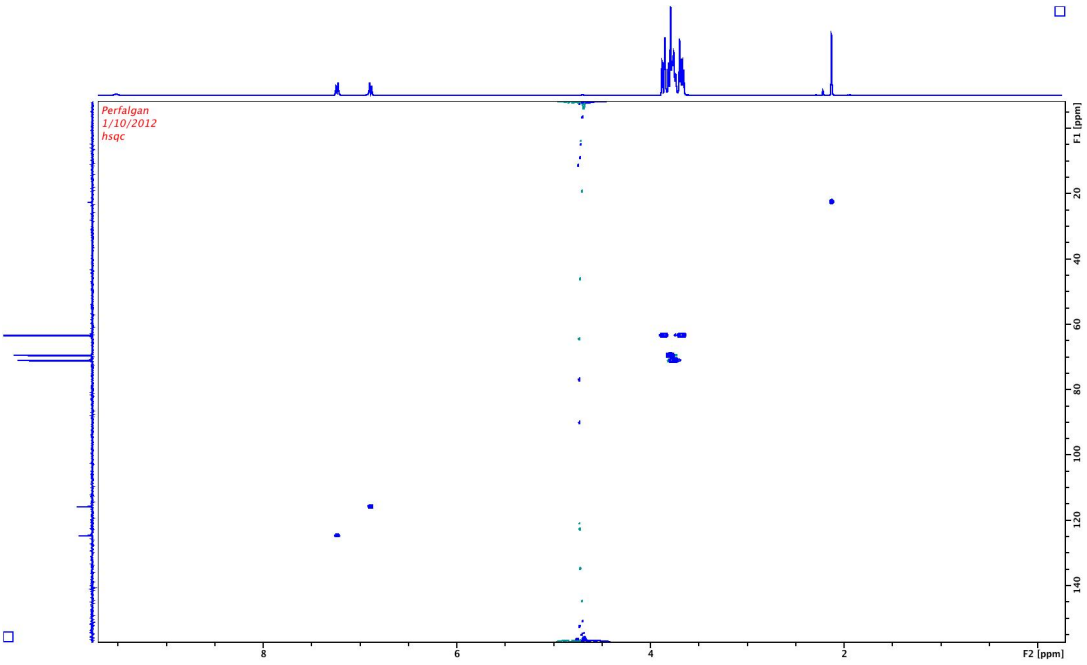


Figure 21: HSQC spectrum of Perfalgan® showing correlation between the ^{1}H and ^{13}C spectra

Summary:

The HSQC spectrum showed no change in the paracetamol and mannitol peaks. The cysteine converted to cystine as discussed before. No new compounds were identified.

10.2.2.3.3 ^{13}C analysis

^{13}C is the only carbon nucleus that is spin active. Unlike ^{1}H NMR, the ^{13}C plot is not quantitative, meaning that the height of the peaks do not correlate to the number of nuclei in the molecule. The numbers depicted above each peak correspond to the assignment of the carbons in the structure above. For example, in the paracetamol peaks, peak 4 corresponds to the fourth carbon

in the benzene ring. Since the peaks for identical carbons superimpose, the notation 2,6 corresponds to the second and sixth carbon.

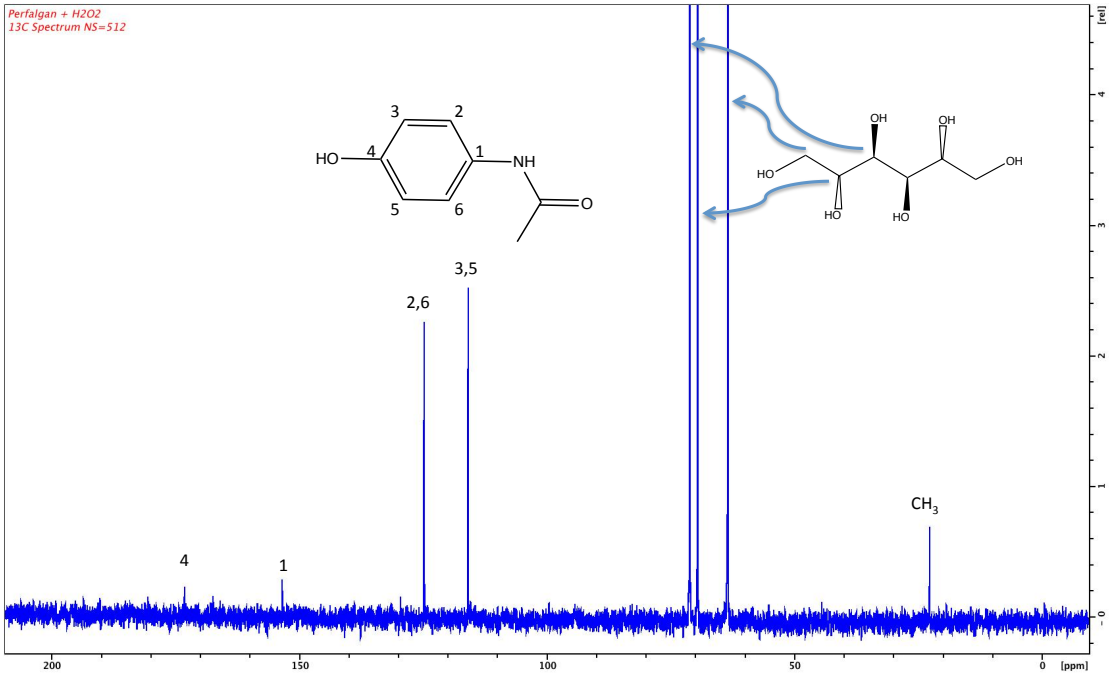


Figure 22: Perfalgan® ^{13}C NMR after treatment with H_2O_2

Summary:

Comparison of Figures 16 and 22 showed no morphological change in paracetamol or mannitol with hydrogen peroxide stress. In particular no new compounds were evident.

10.3 UV spectroscopy results

10.3.1 Paracetamol and Perfalgan® samples

The HPLC instrument used in our study could only look at a single wavelength, which was chosen to be that of paracetamol. Thus there is the possibility that degradation products could have been missed. For this reason ultraviolet spectroscopy was used to assess the samples in the wavelength

range 200 to 800 nm. Of particular interest was whether any colour change occurred within this range which may not be visible to the human eye, but which becomes apparent in Perfalgan® with time. At the same time, since UV can be quantitative, the experiments were also used to check the HPLC quantitation.

Firstly the equipment was calibrated with altering concentrations of paracetamol. The absorbance spectra are shown in Figure 23 below. Note UV/Vis spectroscopy is a very sensitive technique and so the paracetamol had to be diluted in order to give an on-scale reading.

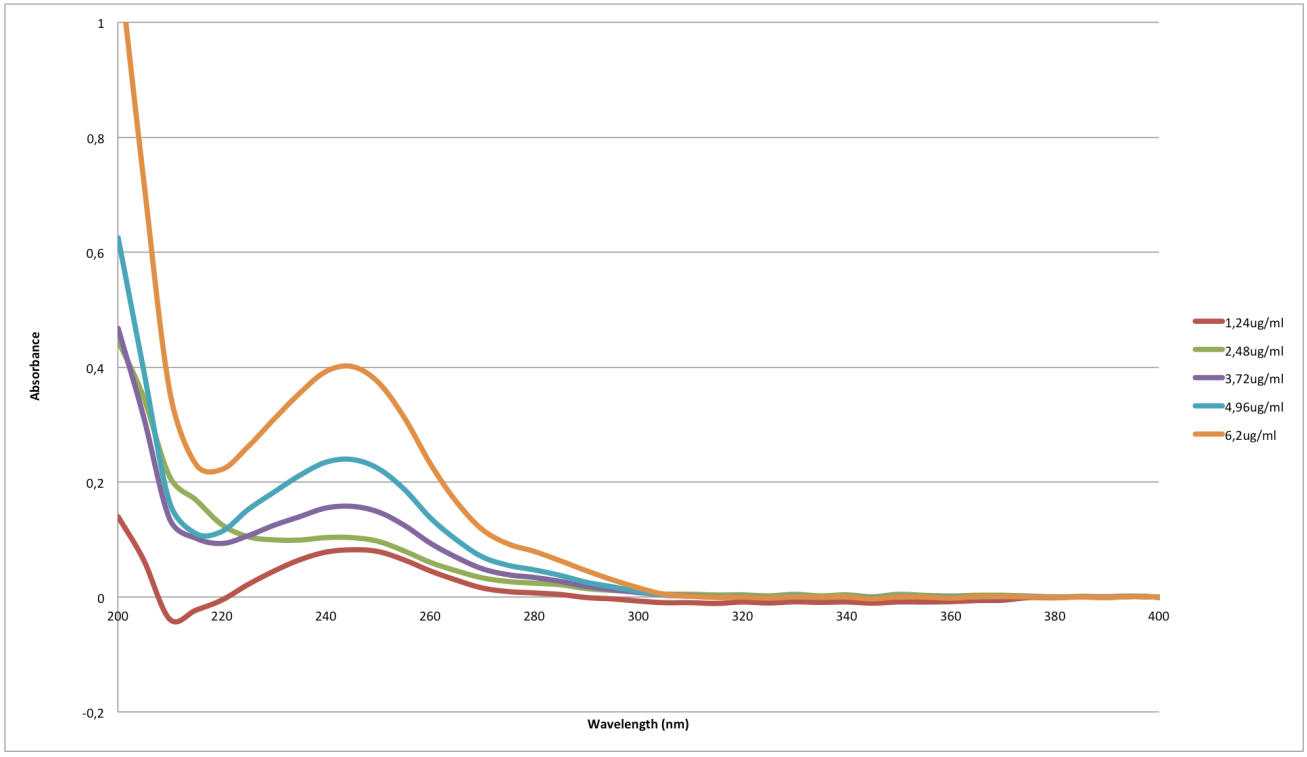


Figure 23: UV absorbance spectra for various concentrations of paracetamol (in µg.ml^{-1})

The wavelength of 245nm was chosen to construct the calibration curve as it correlated with the maximum peak absorbance at each concentration. (cf. Figure 24)

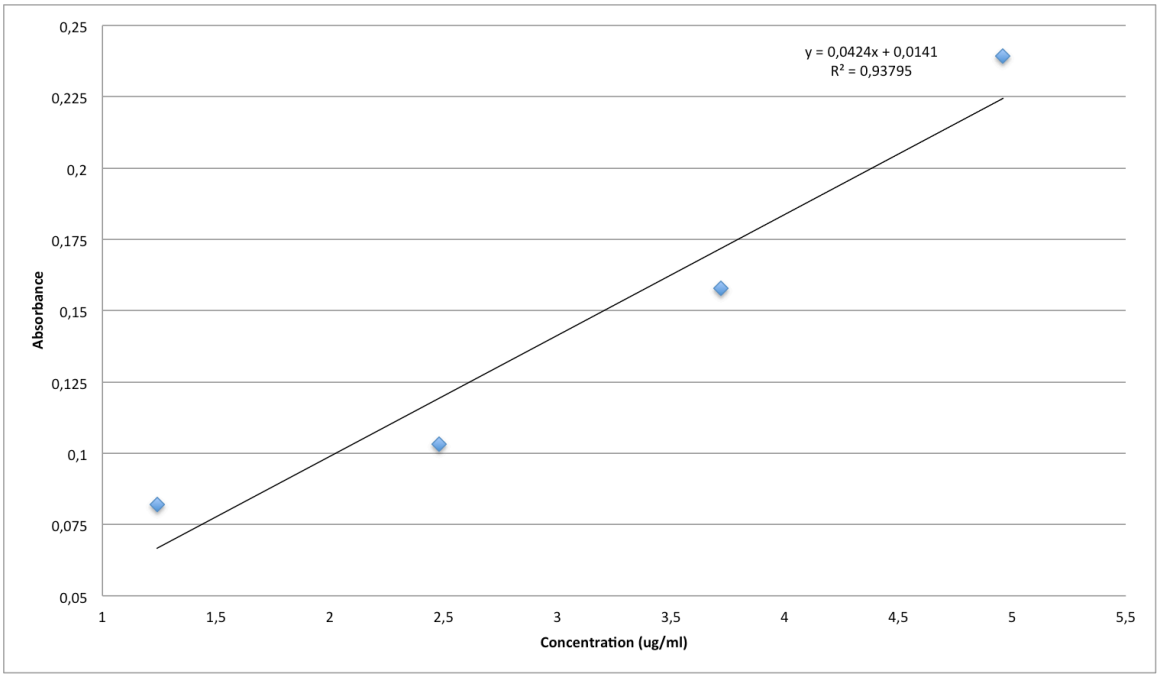


Figure 24: Paracetamol calibration curve for UV spectroscopy measured at 244.96 nm

Table 7: UV spectroscopy paracetamol calibration curve parameters

Correlation coefficient	0.94
Standard error	0.02
Slope	0.04
Standard deviation in slope	0.01
Intercept	0.01
Standard deviation in intercept	0.03

This calibration curve was used to calculate the concentrations of the paracetamol recovered from Perfalgan® when the samples were assessed using UV spectroscopy. The equation of the straight line was y=0.0424x + 0.0141. The R^2 value was 0,94 making it a less precise method than HPLC. The standard error was also greater than the HPLC. Greater accuracy could have been achieved had standard additions been used. For this reason HPLC was used as the main method for quantifying paracetamol. The purpose of UV spectroscopy was to identify other compounds which may have been present in the sample. In particular, given the sensitivity of the technique it was hoped that any coloured impurities would be detected.

10.3.1 The effect of changing the environment of Perfalgan®

Figure 25 displays the spectra for UV absorbance of Perfalgan® under 'stressed' and 'unstressed' conditions.

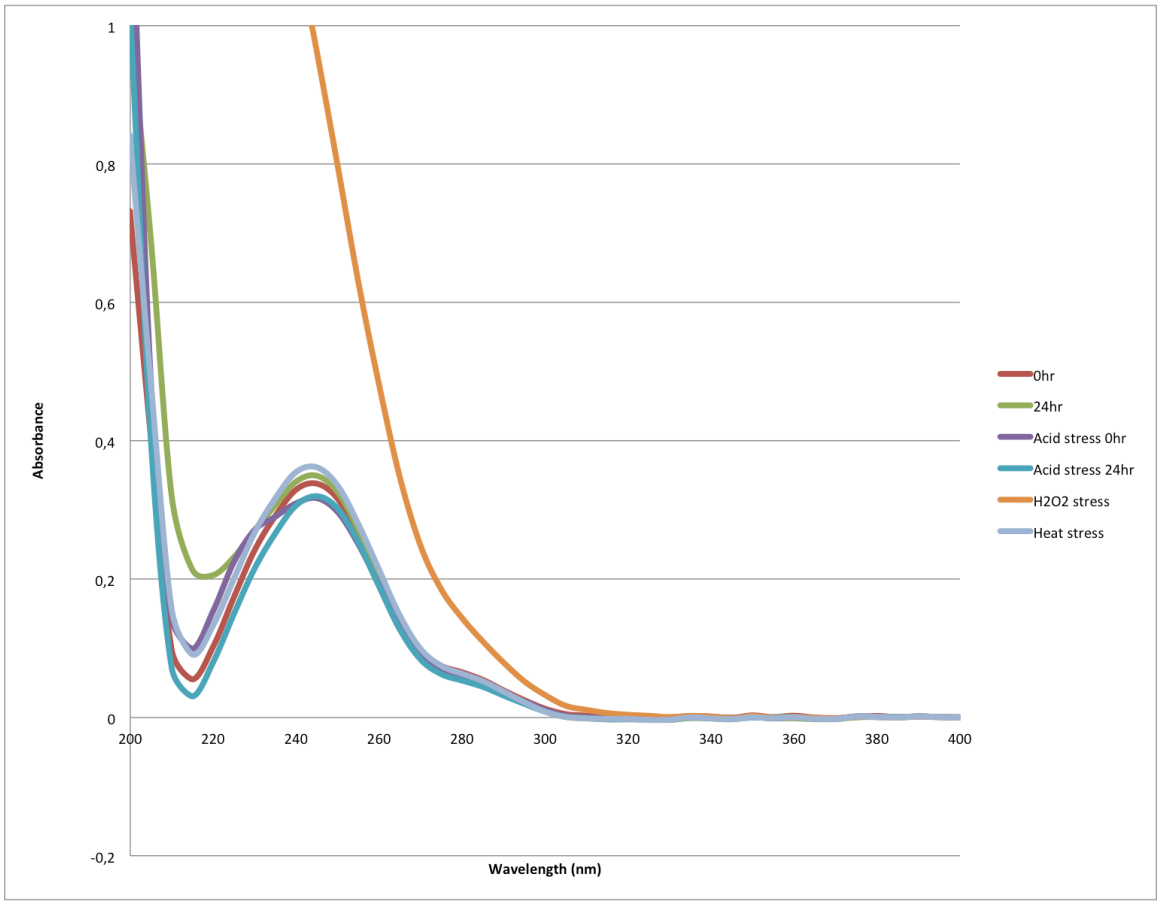


Figure 25: UV spectroscopy spectra for Perfalgan® samples exposed to different environments

From the curves it is clear that there is little if any change in the samples under stress. Using the calibration curve, the concentration of paracetamol of each of the Perfalgan® samples was calculated.

Table 8: Table of absorbances and concentrations of paracetamol (in μg.ml^{-1}) in Perfalgan® samples

	Standard		Acid stress		Heat
	0 hr	24 hr	0 hr	24 hr	24 hr
Absorbance	0.34	0.35	0.32	0.32	0.36
Concentration	7.7	7.9	7.2	7.2	8.2
Standard error	1.0	1.0	0.9	0.9	1.1
95% Confidence interval	4.3	4.5	4.0	4.0	4.6

Summary:

The spectra show minimal alteration in the paracetamol peak on changing conditions.

The concentrations of paracetamol recoverable via UV spectroscopy stayed constant, despite being greater than the 4,76 μg.ml^{-1} expected. This is a result of an incorrect background subtraction. The method of standard additions could have been used to take into account any matrix effect. However, as no change was seen and given that UV is a less specific analytical technique it was decided not to pursue this option. The H_2O_2 has absorbance of its own which precluded examination of the absorption of the Perfalgan® sample containing H_2O_2, as displayed by the orange curve in figure 25.

10.3.1 Perfalgan® octanol:water partition coefficient

10.3.1.1 Paracetamol testing

UV spectroscopy was first performed on paracetamol mixed in octanol to create a calibration curve.

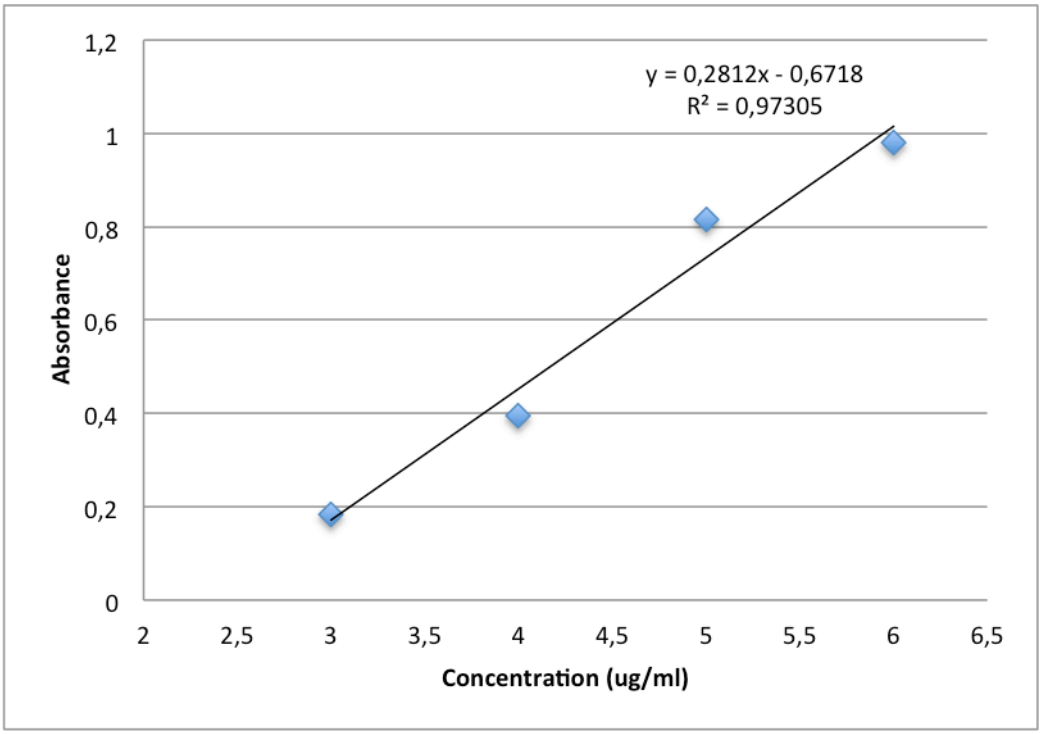


Figure 26: UV spectroscopy calibration curve for 50:50 parcetamol:octanol measured at 250.01 nm

The formula for the straight line was y=0.2812x-0.6718. The R^2 value is 0.97 making it an accurate calibration curve.

Table 9: UV spectroscopy 50:50 paracetamol:octanol calibration curve parameters

Correlation coefficient	0.97
Standard error	0.07
Slope	0.28
Standard deviation in slope	0.03
Intercept	-0.67
Standard deviation in intercept	0.15

10.3.1.2 Perfalgan® testing

UV spectroscopy was then performed on 'stressed' and 'unstressed' Perfalgan® samples mixed with octanol. The samples were shaken. Since Perfalgan® is formulated in water, the octanol formed a layer above the aqueous layer. Each layer was analysed for paracetamol, as depicted in Figures 27 and 28 below. (In the key 'pf' represents Perfalgan®.)

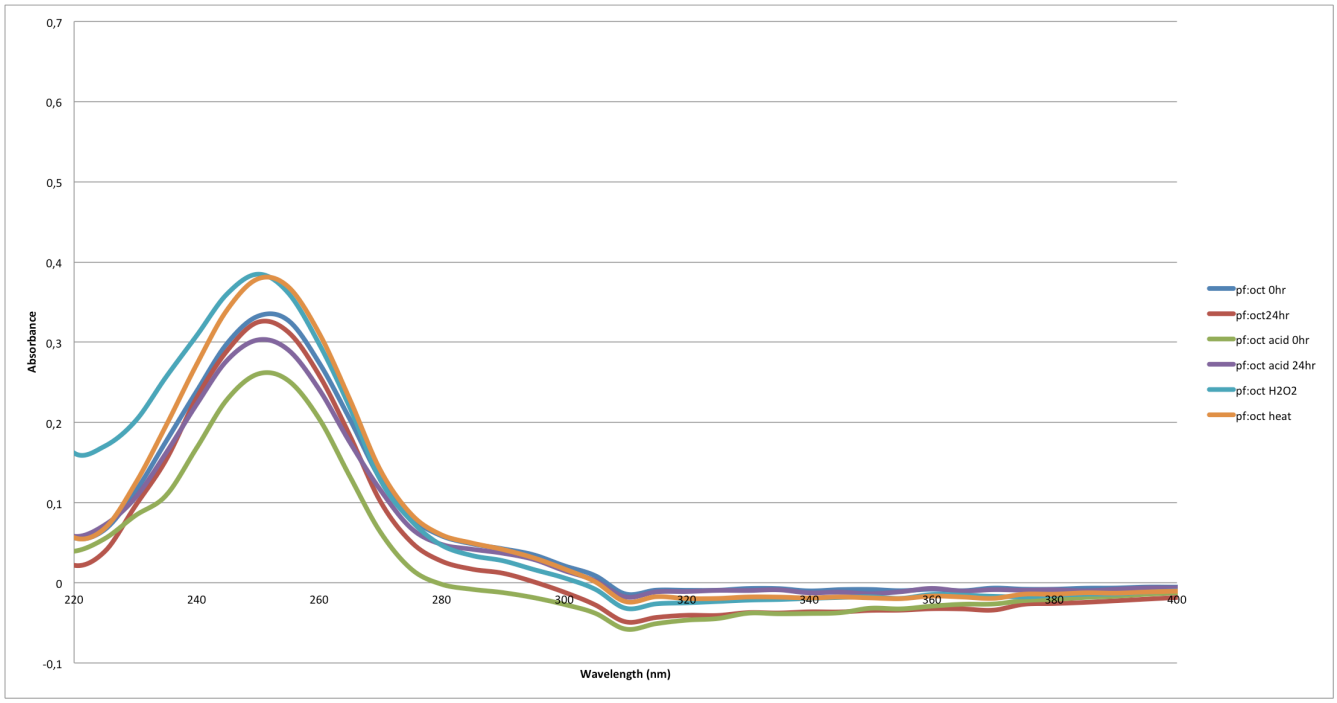


Figure 27: UV spectrum of the organic layer in 50:50 Perfalgan®:octanol

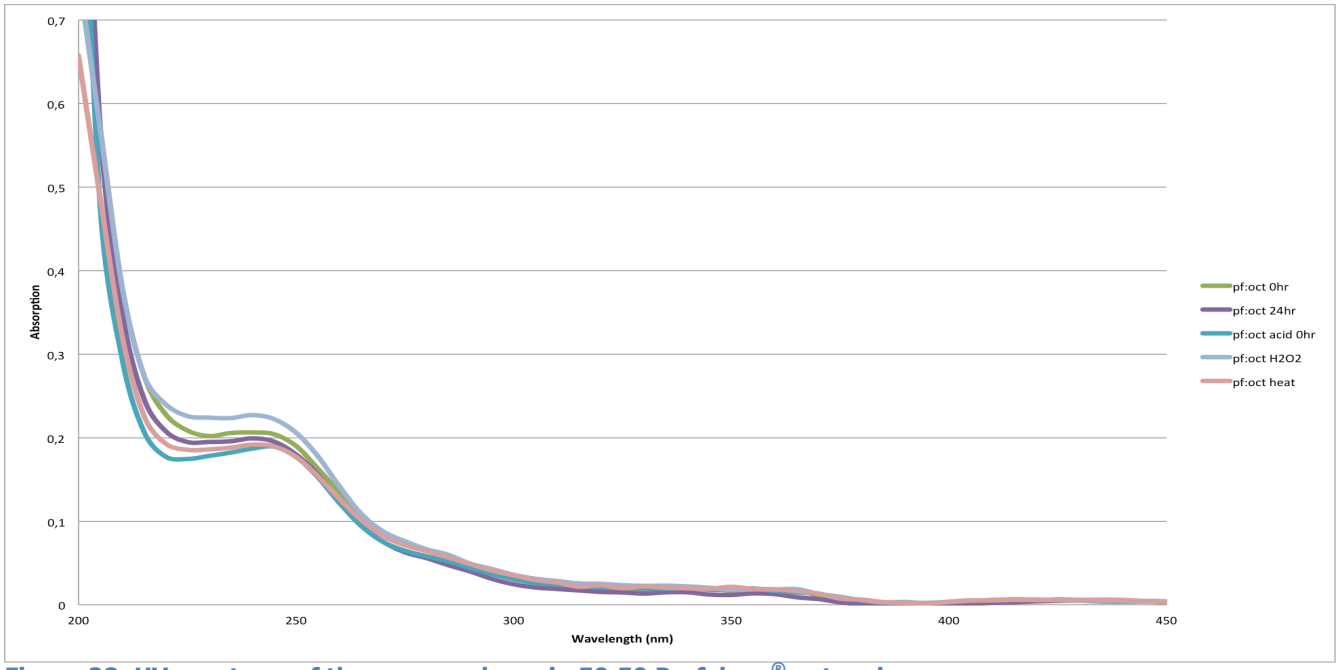


Figure 28: UV spectrum of the aqueous layer in 50:50 Perfalgan®:octanol

Using the paracetamol:octanol calibration curve it was possible to calculate the concentration of paracetamol recovered in each Perfalgan® sample. The concentration in the organic (or octanol) layer was subtracted from the known total concentration of 4.76µg.ml^{-1} in the stock solution to give the concentration in the aqueous layer. Although measurements of each layer (i.e. organic

and aqueous layer) could have been performed it is common practice to measure the concentration of a drug in one layer and calculate the concentration in the other. The ratio of these two values is the octanol:water partition coefficient. The coefficient is often also given as the log of this value. The samples show good agreement with the literature where the partition coefficient ($logP_{ow}$) of paracetamol is quoted as 0.46-0.49 (International Progamme on Chemical Safety, 2008; McNeil Consumer Healthcare, 2010).

Table 8: Comparison of absorbances of various Perfalgan® samples mixed with octanol

	pf:oct 0hr	pf:oct24hr	pf:oct acid 0hr	pf:oct acid 24hr	pf:oct H2O2	pf:oct heat
UV absorbance of organic layer	0.33	0.33	0.26	0.30	0.39	0.38
UV absorbance of aqueous layer	0.18	0.19	0.18	0.18	0.67	0.21
Ratio of absorbances (organic:aqueous)	0.27	0.23	0.16	0.24	-0.24	0.27
Concentration of paracetamol in organic layer (in $\mu g.ml^{-1}$)	3.57	3.55	3.32	3.47	3.76	3.74
Concentration of paracetamol in aqueous layer (in $\mu g.ml^{-1}$)	1.19	1.22	1.45	1.30	1.01	1.03
Octanol:water partition coefficient	2.99	2.91	2.29	2.67	3.73	3.64
$logP_{ow}$	0.48	0.46	0.36	0.43	0.57	0.56

* Calculations were performed on numbers to three decimal places. This table shows only the first two decimal places.

Summary:

The concentration of paracetamol penetrating the lipid or aqueous layer remained constant despite stress applied to the Perfalgan® samples over 24hr. This implies that bioavailability was unaltered.

11 DISCUSSION

Each method of analysis showed a well-defined peak for paracetamol. The retention time using HPLC was in the range of 3.2 to 3.3 minutes, which correlated well with that found in the literature. Although subjected to extreme conditions such as acid, alkali, heat and oxidative stress, the paracetamol in Perfalgan® did not degrade.

A variety of methods was used to analyse Perfalgan® in this study. While HPLC was more accurate than UV spectroscopy (with an R^2 value of the calibration curve of 0,98 as opposed to 0,94 and smaller standard error) it was limited by the fact that it only analysed paracetamol at one wavelength, 266 nm. Both methods showed that paracetamol did not degrade significantly over a 24 hour period on exposure to air. There was therefore no need to analyse samples drawn at hourly intervals.

11.1 HPLC results

The paracetamol in Perfalgan® did not degrade significantly over a 24 hours period on exposure to air. Of the stressed samples, the paracetamol in heated Perfalgan® was unchanged. The Perfalgan® was found to precipitate in base precluding further analysis with HPLC. Filtered samples showed very small peaks at the same retention time due to loss of paracetamol by the filtering process. The precipitation may be the paracetamol alone or contain buffer from the mobile phase. Further studies with different mobile phase would need to be done to elucidate this.

Acid-stressed Perfalgan® samples showed a paracetamol peak at the same retention time but with reduced area under the curve. There is little difference between the 0 hour and 24 hour acid samples. Again, this was due to precipitation of the active ingredient when subjected to acid in a methanolic solution. Since the peak is at the same retention time and shape, it is unlikely to be due to paracetamol degradation.

Subjecting Perfalgan® and paracetamol stock solution to hydrogen peroxide gave a double peak in the HPLC chromatogram. Two peaks could be due to two different compounds eluting with similar retention times i.e. one peak is paracetamol and the other is a degradation product. Since this did not occur with UV spectroscopy or NMR, it may be concluded that this is not two peaks but a splitting of the paracetamol peak. Split peaks are known to occur in HPLC because the injected volume or sample concentration is too high. Since the same injection volume and sample concentration was used in all the experiments this is unlikely to be the cause of the split peak. Similarly we can discount a column void or channeling as the reason for the peak splitting. The most likely reason is a polarity difference between the mobile phase and injection solvent. The high concentration of peroxide in the injectate relative to the mobile phase caused the peak to split. It should be mentioned that splitting of the peak occurred originally when pure Perfalgan® was injected, therefore methanol was added to the samples to more closely match the polarities. It was decided not to pursue the cause of the split peaks further as it was not part of the main objectives of the study.

Quantitative analysis of the paracetamol in Perfalgan® proved difficult using HPLC due to precipitation in the acid- and base-stressed samples and splitting of the peak as in the case of H_2O_2 exposure. However, qualitative analysis showed that samples had the same tR and no degradation products of paracetamol were seen.

11.2 ^{1}H NMR results

NMR showed Perfalgan® to be stable. The paracetamol peak was unaltered in each sample. Of its excipients only cysteine changed under the various tests performed. On exposure to air the cysteine oxidised to cystine. This process took 48 hours under 'unstressed' conditions, but was much faster under 'stress', ie 12 hours on heating and 1 hour on addition of H_2O_2 respectively. The Perfalgan® package insert lists cysteine as an anti-oxidant that is added to protect the paracetamol from oxidative stress. The literature also mentions that some samples of Perfalgan® may have a slight yellow colour. This did occur in one of our samples when stressed with peroxide. However the source of the colour could not be identified with any of the analytical techniques used in this

study. It was noted, however, using ^{1}H NMR that there were minor impurities in the Perfalgan®. However these impurities were at a low level and could not be identified.

11.3 UV spectroscopy results

UV spectroscopy again showed a constant concentration of paracetamol over time and on exposure to stressors. On calculating the concentration of paracetamol in each sample, the values were much higher than expected, presumably due to another species in the vial absorbing at the same wavelength. The method of standard additions should have been used to quantify the paracetamol rather than the calibration curve. Since H_2O_2 has its own absorbance it was not possible to assess the peroxide-stressed Perfalgan® samples using this method.

11.4 Octanol:water partition coefficient

The concentration of paracetamol in the octanol (or organic layer) remained constant despite stress applied to the samples. $LogP_{ow}$ was 0.48 for Perfalgan® at 0 hr and did not change significantly over time and under 'stress'. Although being calculated and not directly measured, this value shows good agreement with the literature, which quotes the figure between 0.46-0.49. The $logP_{ow}$ values for H_2O_2- and heat treated Perfalgan® were about 20% greater than unstressed. $LogP_{ow}$ values of <1 indicate high solubility, 1-3 moderate solubility and 3-5 low solubility. Within this context it is clear that this increase does not have a significant impact on the drug's solubility.

12 CONCLUSIONS

In summary, the findings of this study were that with three different assay methods the paracetamol in Perfalgan® was stable with regards to its structure, concentration and bioavailability over a 24 hour period and despite changing its environment. In answer to the research question put at the beginning of the study, it follows that the practice of using a single vial of Perfalgan® multiple times over the course of a day will render a constant concentration of paracetamol, of equal bioavailability at the end of the day relative to a newly opened vial. The practice of using Perfalgan® as a mutidose vial is therefore valid from a chemical point of view.

Since this practice is mostly used in the paediatric setting, it has potential cost-saving implications for paediatric anaesthesia and analgesia. Concerns about underdosing children from a previously opened vial are invalid.

Sterility may be a legitimate barrier to the clinical application of this process. While this study assumes that the clinician uses a sterile hypodermic needle and alcohol swab before re-puncturing the vial to withdraw the desired dose, no sterility tests have been performed on the vial and contents once opened. Further microbiological testing therefore should to be performed before this practice can be fully endorsed.

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Permission was granted by Associate Professor Neil Ravenscroft to use the High Performance Liquid Chromatograph and the ^{1}H NMR at the University of Cape Town, Department of Chemistry Laboratory. He and Professor Graham Jackson supervised the analysis. The department covered the analysis costs.

Perfalgan® was purchased privately. Pure paracetamol was obtained from the University of Cape Town, Department of Inorganic Chemistry.

Bristol-Myers Squibb, the distributors of Perfalgan®, had no involvement in the study, financial or otherwise.

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