

Theobromine as a potential cough suppressant biology essay

[Science](#), [Biology](#)



ABSTRACT

Background

Theobromine an extract of cocoa beans has been discovered to have cough suppressant and tooth enamel strengthening properties. Theobromine has a reported solubility in water of only 1g in 2000ml (very slightly soluble) but has been reported to be well absorbed from the GI tract and has also been reported to be absorbed faster in chocolate form compared to capsule form.

Aim

The aim of this project was to investigate the solubility of theobromine, in particular its solubility at pHs of the stomach and small intestine. And to investigate why theobromine is absorbed faster in a chocolate form compared to a capsule form.

Methods

Theobromine's solubility at acidic, neutral and alkaline pHs was investigated. The effect of various surfactants on theobromine's solubility was also investigated.

Results

Solubility investigated at acidic pH of stomach and neutral pH of intestines showed theobromine to have a solubility of what is reported in water of 0.5mg per ml. The extent of solubility at acidic and neutral pH was found to not increase over time. There was little to no change in solubility with the surfactants; sodium deoxycholate, SDS and CHAPS.

Conclusion

The findings suggest theobromine to have a solubility of around 0.5 mg per ml when in the GI fluids. And as the rate and extent of solubilisation did not differ greatly between pH 2 and pH 7, it seems unlikely that differences in the amount of theobromine solubilised with residence time could explain differences in absorption with or without food. And the little to no effect of sodium deoxycholate on theobromine's solubility suggests bile acids might not have much effect on theobromine's solubility.

Significance and impact of study

With theobromine potentially being developed as a cough suppressant at high doses this work on its solubility may be useful for those formulating theobromine as a cough medicine.

INTRODUCTION

1.1. Theobromine's therapeutic discoveries.

Theobromine, an extract of cocoa beans, is found in products which are consumed regularly by people all over the world. Following research into theobromine, it has been found to have cough-reducing and tooth enamel-strengthening properties. Following this discovery, it has been developed into a toothpaste named 'Theodent' with theobromine as the main active ingredient instead of fluoride (Theodent, 2012). Theobromine's mode of action is said to involve increasing the size of crystals within the bone structure, suggesting a possible use of theobromine in bone development (Nakamoto et al., 1999). It is also believed to suppress cough by inhibiting

sensory nerve activation at relatively high concentrations (Usmani et al., 2005).

1. 2. Theobromine as a potential cough suppressant

1. 2. 1. Cough in general

Cough is a protective reflex mechanism which aids in clearing the upper airways from mucus, harmful substances, foreign particles and infectious organisms, preventing them from entering the lower respiratory tract (Chung and Pavord, 2008). The importance of cough as a protective reflex mechanism is emphasized by the complications which result as a result of cough suppression after general anaesthesia; problems that arise include infections and holding of airway secretions (Chung and Pavord, 2008). The cough reflex results from stimulation of cough receptors of which two classes exist. These are known as rapidly adapting receptors (RARs) and slow adapting receptors (SARs- also known as C-fibre receptors) (Mazzone, 2005). RARs can be stimulated by a wide range of factors which would result in the cough response. Stimuli of RARs include cigarette smoke, dust, mucus, acidic and alkaline conditions, hypertonic and hypotonic saline, mechanical stimulation and decrease in lung compliance (Chung and Pavord, 2008). SARs are more sensitive to chemicals which is why they are also known as chemosensors. They are highly sensitive to chemicals such as bradykinins, capsaicin and hydrogen ions (Mazzone, 2005). Although cough is a protective reflex mechanism in healthy persons but when it becomes persistent and doesn't benefit the individual it is one of the most common health complaints (Usmani et al., 2005). Short term coughs from bacterial or viral

infections are common health complaints doctors come across, these coughs are usually self-limiting. Common causes of chronic cough (greater than 8 weeks) include cigarette smoking as well as exposure to cigarette smoke. Chronic coughs can also be caused by diseases such as asthma, chronic obstructive pulmonary disease (COPD), gastroesophageal reflux disease and cancer (Chung and Pavord, 2008). In the US up to 80% of lung cancer patients and around 37% of all cancer patients suffer from chronic coughing. In the England and Wales there are 900, 000 people diagnosed with COPD but from the results of a survey the number could be around 4 million (healthandsafetyexecutive, 2012). A great number of people suffer from persistent coughing, which can seriously affect ones quality of life (Usmani et al., 2005). Persistent cough can be socially distressing, can cause sleeplessness and fatigue (Halfdanarson and Jatoi, 2007). In extreme scenarios coughing can also cause rib fractures (Halfdanarson and Jatoi, 2007). Individuals suffering from persistent cough commonly resort to over the counter medicines which have led to annual sales in the US of over 2 billion dollars (Usmani et al., 2005). However a meta analysis done on cough remedies commonly bought over the counter found that there was inconclusive evidence on their effectiveness (Usmani et al., 2005). Commonly bought cough remedies such as codeine and dextromethorphan have unpredictable efficacy, furthermore they have central and peripheral side effects which usually results in individuals discontinuing them (Usmani et al., 2005). This highlights the need for new and effective cough treatments.

1. 2. 2. Theobromines antitussive findings by Usmani et al.

Findings by Usmani et al. indicate theobromine to be a novel and promising treatment of cough (Usmani et al., 2005). Usmani et al. have carried out various experiments to investigate theobromines cough suppressant properties. In one experiment they investigated if theobromine would inhibit cough induced by citric acid in guinea pigs. They found theobromine and codeine, which was used as a positive control, had similar antitussive effects. They also found theobromine given at a dose of 32mg per kg resulted in cough suppressant effects for up to four hours (Usmani et al., 2005). Furthermore in an experiment on 10 human volunteers Usmani et al. investigated if theobromine could inhibit cough induced by capsaicin. Capsaicin is commonly used to induce cough in clinical research. The volunteers were given either a 1000mg dose of theobromine, a 60mg dose of codeine or a placebo. The findings revealed theobromine increased the amount of capsaicin that was needed to induce five coughs in comparison to codeine and placebo (Usmani et al., 2005). These findings show the potential of theobromine as an antitussive especially as it out performed codeine which is a commonly used antitussive.

1. 2. 3. Current clinical trials

Clinical trials investigating the antitussive effects of theobromine have been undertaken in 2011 in the Republic of Korea where individuals were administered with 300mg theobromine capsules. The findings have not yet been posted on the clinicaltrials.gov website. More recently at thirteen NHS hospitals around 300 individuals who suffer from persistent cough took part

in clinical trials to investigate the effects of theobromine on cough. In the trial individuals were given 1000mg capsules of theobromine two times a day for a period of 14 days (DailyMail, 2012) . The findings were presented in December 2012 at the British Thoracic Society's winter meeting. They found more than half of the patients experienced some relief but this relief was lost once theobromine was stopped. The principal investigator said a eating a bar of dark chocolate daily may be effective for people who suffer from chronic cough (Renter, 2013).

1. 3. Theobromines use in toothpaste

1. 3. 1. Tooth enamel

Teeth are composed of many parts which include the enamel, dentin, pulp and root. The enamel makes up the outer layer of tooth and it is believed to be the hardest and the most mineralized tissue in the body (colgate, 2011). The main mineral which makes up the enamel is known as hydroxyapatite, it has the formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. In reality the mineral phase of the enamel is not a pure form of hydroxyapatite but a modified version of this mineral known as carbonated calcium deficient hydroxyapatite (Lussi et al., 2012). The OH ion of the apatite can be exchanged with ions, for example OH could be replaced with fluoride to make fluoroapatite (Lussi et al., 2012). Generally in human's the most outer layer of the enamel, has less than 5% (on average) of the OH groups of hydroxyapatites replaced with fluoride. And the percentage of OH groups replaced with fluoride decreases as you go deeper into the enamel (Lussi et al., 2012).

1. 3. 2. Tooth decay

Although the minerals in the enamel give the enamel its hardness they are vulnerable to demineralization which results in dental diseases such as tooth decay. Plaque containing bacteria form on the teeth and after a meal containing sugars the bacteria feed on the sugars and produce acids. The acids produced then attack and destroy the enamel (Webmd, 2011). A common advice which is given to prevent tooth decay is the use of fluoride toothpaste. Fluoride toothpastes are believed to result in fluorapatites which are bigger crystals and believed to be more resistant to acid attacks compared to hydroxyapatites (scientificamerican, 1999).

1. 3. 3. Alternative to fluoride based toothpaste

The majority of toothpastes contain fluoride however the safety of the use of fluoride in toothpaste has been debated. A side effect said to result from the use of fluoride is dental fluorosis which can result in erosion and staining of the enamel. There is also a risk of poisoning from toothpaste which has led to the FDA enforcing toothpaste companies to place the warning 'Keep out of reach of children under 6 years of age. If you accidentally swallow more than used for brushing, seek professional help or contact a poison control centre immediately' (FluorideActionNetwork, 2013).

1. 3. 4 Discovery of the benefits of theobromine

With the majority of toothpaste being fluoride based and issues regarding their safety indicates the need for safer alternatives. Alternatives so consumers have more choice in toothpastes. And with the discovery by Nakamoto et al. an alternative has been found. After multiple years of

research studying methylated xanthines such as theobromine, paraxanthine and theophylline. It has been discovered theobromine has beneficial use in the area of bone and dental diseases (Nakamoto et al., 1999). This idea has progressed to an actual product, a patented toothpaste called 'theodent' which is available for purchase by the general public (Theodent, 2012). In vitro experiments by Nakamoto et al. into the effects of theobromine on apatite crystals found bigger crystals were formed in the presence of theobromine compared to crystals formed in the absence of theobromine. The bigger crystals can possibly result in increased hardness and a greater resistance to acid dissolution. Experiments on human teeth comparing theobromine directly with fluoride have also been carried out. Making the discovery that theobromine showed to make teeth harder and less susceptible to acid dissolution compared to fluoride (Nakamoto et al., 1999). This discovery and product has led to widespread media coverage more recently in the daily mail making the headline 'Remarkable discovery leads to chocolate toothpaste that is actually GOOD for your teeth' (DailyMail, 2013). This discovery of theobromine forming larger crystals could lead to further treatments in the area of bone and dental disease.

1. 4. Solubility

1. 4. 1. Solubility

For drugs to be absorbed across biological membranes they must first be in solution. So when drugs are given in solid form such as powders they must first solubilise before they can be absorbed unless the drug is given as a solution. However even if the drug is given as a solution it may come out of

solution for example in the stomach and so would have to dissolve again before it is absorbed (Aulton and Ashford, 2002). The solubility of a drug is the amount of drug which goes into solution to produce a saturated solution at a specific temperature and pressure (Aulton and Ashford, 2002). The extent of solubility (i. e. amount of drug going into solution) varies considerably between the various existing drugs with some being highly soluble whilst others having a poor solubility (Savjani et al., 2012). Solubility can be expressed in many ways such as in molality, molarity, mole fraction and parts (Savjani et al., 2012). But probably the most common expression of drugs solubility would be the amount (mass or volume) of drug (solute) dissolved in a certain volume or mass of solution at a specific temperature, for example a drugs solubility in water could be expressed as 10mg per 100ml (Aulton and Ashford, 2002). The following table shows how the solubility of drugs can be classified; this classification is used in the US and British pharmacopeia's (Aulton and Ashford, 2002). Weight of solvent (in grams) needed to dissolve one gram of drug

Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10000
Practically insoluble	More than 10000

(Aulton and Ashford, 2002)

1. 4. 2. Biopharmaceutical classification system

Drugs can be classified in the biopharmaceutical classification system into four categories. Drugs are classified according to two parameters; their solubility over GI fluids pH range (pH 1 to pH 7. 5) and their permeability across the GI tract (Aulton and Ashford, 2002). Highly soluble drugs are one

in which the highest dose of the drug can be dissolved in a volume of 250ml over a pH range 1 to 7.5 (Martinez and Amidon, 2002). Highly permeable drugs are ones which have 90% or more of the administered dose absorbed (Aulton and Ashford, 2002). Categorising drugs according to their solubility and permeability allows educated predictions to be made as to how variables such as food, formulation and disease states can affect the bioavailability of drugs (Martinez and Amidon, 2002). The four classes of the biopharmaceutical classification system are shown below; Class 1- high solubility, high permeability Class 2- low solubility, high permeability Class 3- high solubility, low permeability Class 4 low solubility, low permeability (Martinez and Amidon, 2002)

1. 4. 3. Factors affecting solubility

There are many factors which can influence a drug's solubility in solution. Factors include the shape and hydrophobicity of the drug molecule, the drug's pKa, its ionisation state and the pH of the medium (Aulton and Ashford, 2002). The drug's pKa and the GI fluids pH can affect solubility as it will influence the percentage of drug ionised and percentage unionised (providing the drug has ionisable groups). This parameter is important to consider as the GI tract's pH varies from around pH 1 to pH 7.5. The ionised form of the drug is generally more soluble than the unionised form. To help determine the percentage ionised at a specific pH the following equations can be used (Dr Freeman, 2013). For a weak acid: percentage unionised = $\frac{1}{1 + 10^{pH - pK_a}}$ For a weak base: percentage unionised = $\frac{1}{1 + 10^{pK_b - pH}}$ The solubility of weak acids and weak bases will vary depending on where they are in the gastrointestinal

tract. Drugs which are weak bases will have more drug in ionised form at acidic pH and so more soluble at acidic pHs whilst weak acids will be more soluble at higher pHs. And so drugs which are weak bases will be more soluble in the stomach whilst drugs which are weak acids will be more soluble in the intestine (Martinez and Amidon, 2002).

1. 5. Dissolution

1. 5. 1. Dissolution

Dissolution is the process when molecules or ions of a solid state are transferred into solution. This is an important parameter to consider as drugs must dissolve before they can be absorbed (Martinez and Amidon, 2002).

The factors which can affect the rate of dissolution of a solid drug can be determined by considering the terms of the Noyes-Whitney equation (Aulton and Ashford, 2002). Noyes Whitney equation: = Rate of dissolution $D = \frac{D_s A (C_s - C)}{h}$
Diffusion coefficient D = Surface area of drug particle exposed to the GI fluid A = Diffusion layer thickness h C_s = Solubility of drug in solution C = Drug concentration in GI fluids (Aulton and Ashford, 2002)

1. 5. 2. Factors affecting dissolution rate

1. 5. 2. 1. Physiological factors

The parameters of the Noyes-Whitney equation can be directly influenced by the physiology of the gastrointestinal tract. For example surfactants are released in the gastrointestinal tract. Surfactants in the gastrointestinal tract can affect the solubility of the drug through micelle formation. Furthermore as surfactants affect the wettability of drug particles they will as a result affect parameter A which is the surface area of drug particle exposed to the

GI fluids. The thickness of the diffusion layer is affected by how much agitation each drug particle experiences and so the level of agitation in the GI tract will affect the dissolution rate. The amount of drug in solution in the GI fluids (parameter C) will be affected by a number of factors. It will be affected by how fast or slow the drug in solution is absorbed. Parameter C will also be influenced by how much fluid the drug is exposed to for dissolution, the more fluid the drug is exposed to the more drug will go into solution. The amount of fluid the drug is exposed to will be influenced by the diet and the position within the gastrointestinal tract (Aulton and Ashford, 2002). Furthermore the degree of agitation experienced by the drug in the gastrointestinal tract will affect the thickness of the diffusion layer (parameter h). The greater agitation experienced by the drug will result in a smaller diffusion layer and so increase the dissolution rate. Parameter D, which is the diffusion coefficient, can be influenced by food because food can increase the viscosity of the fluid. The increased viscosity would decrease the rate at which drug molecules would move away from the diffusion layer and so the rate of dissolution would be decreased (Aulton and Ashford, 2002).

1. 5. 2. 2. Drug factors

The drug itself can also have an influence on the rate of dissolution for example the size of the drugs particles. A smaller sized drug particle will result in a greater surface area of the drug particle exposed to the fluids of the GI tract and so a greater dissolution rate. And so when formulating a drug it can be purposely made to a small size with the intention of increasing

the drugs dissolution rate and to even increase the drugs bioavailability if the rate limiting step for absorption is the dissolution step. Rate of dissolution is also dependent on the solubility of the drug in its diffusion layer and in the case of weak acids and weak bases this solubility will be affected by the pH of the surrounding GI fluid. Weak acids will have a greater solubility at higher pH's hence a greater solubility in the intestine relative to the stomach. The opposite would be true for weak bases which will have a greater solubility at acidic pH and so will have a greater solubility in the stomach compared to the intestine (Aulton and Ashford, 2002). As weak acids show greater solubility at higher pHs and so would be poorly soluble in the stomach because of the acidic pH, the pH of the diffusion layer can be raised through the formation of salts. In this case the pH of the diffusion layer when the drug is in the stomach would be around 5 or 6 which is higher than the pH of the stomach. Because of the increased pH of the diffusion layer this will result in a greater dissolution rate(Aulton and Ashford, 2002).

1. 6. Absorption

1. 6. 1. Brief overview of the gastrointestinal tract

The gastrointestinal tract is about six metres in length going from the mouth to the anus. It can be separated into the four regions. Firstly the oesophagus, then the stomach which is then followed by the small and large intestines (Aulton and Ashford, 2002). The oesophagus basically connects the mouth to the stomach moving content taken via mouth into the stomach. Once the content reaches the stomach, it is worked on by acids and enzymes in the stomach to produce what is known as chyme. Chyme has a creamy

consistency which helps absorption of ingested contents once in the intestine. Stomach has a capacity of around one and a half litre and has an acidic pH ranging from pH 1 to pH 3.5, this acidity is due to the secretion of acids by parietal cells. Although drug absorption occurs in the stomach this is relatively very small in comparison to the drug absorption in the small intestine. This is because the small intestine has a much larger surface area than the stomach. The stomach also acts as a reservoir storing ingested content before passing the content on to the duodenum. The rate at which the stomach passes content on to the duodenum i. e. the gastric emptying rate can influence the rate drug absorption (Aulton and Ashford, 2002). The small intestine is where most of the ingested content is absorbed. Although it has a length of about four to five metres it has an enormous surface area of around 200 metres squared, similar to the size of a tennis court. This is because of structural features such as villi and microvilli. Unlike the stomach the intestine has more of a neutral pH of around 6 and 7.5. The small intestine is followed by the large intestine which doesn't have as great of a surface area like the small intestine. It plays an important homeostatic role being involved in the absorption of water and the ions sodium and chloride (Aulton and Ashford, 2002).

1. 6. 2. Food effects on drug absorption

The rate and extent of absorption can be affected by food in the gastrointestinal tract. Food can affect absorption in many ways. It can affect absorption by the formation of complexes. Components of the diet may bind to the drug and this binding may be irreversible or reversible. Drug

absorption would mostly be affected if the drug and food component irreversibly bonded to each other (Aulton and Ashford, 2002). For example the absorption of alendronate is reduced by almost all food including coffee and orange juice. Consequently it is advised it is taken with only water and at least thirty minutes away from anything other medicines or food (Yaheya and Ismail, 2009). Furthermore the pH of the gastrointestinal tract can be influenced by the presence of food. Food generally increases the pH and so the rate of dissolution and absorption of weak acid and weak basic drugs will most likely be affected. Food also affects gastric emptying time, increasing in the presence of food. This will increase or decrease rate of dissolution depending on the drugs properties, for example if a drug was dissolution rate limited then a delayed gastric emptying would increase dissolution. However if a drugs dissolution step wasn't the rate limiting step then delayed gastric emptying would only delay absorption (Aulton and Ashford, 2002). Food could also compete with drugs for absorption, for example if a drug and a food component were absorbed by the same absorption mechanism. Food increases the viscosity of the fluid in the gastrointestinal tract. The increased viscosity often results in a lower bioavailability because an increased viscosity reduces the dissolution rate and decrease the rate at which dissolved drug moves from the lumen to the absorption site (Aulton and Ashford, 2002). Food can also affect the bioavailability by interfering with a drugs metabolism by either inducing or inhibiting the enzyme which metabolises the drug. A common example is the effect of cranberry juice on warfarin biavailability. Cranberry juice inhibits the enzymes responsible for the metabolism of warfarin and so increases its bioavailability. Food also

affects the blood flow to the gastrointestinal tract and the liver by increasing the blood supply after a meal. In the case of drugs which are vulnerable to first pass metabolism an increased blood supply to the liver can increase their bioavailability. This is because as there is an increased blood supply to the liver a higher percentage of the drug escapes the metabolising enzymes (Aulton and Ashford, 2002).

1. 7. Theobromine

1. 7. 1 Theobromine profile

Theobromine is a methyxanthine found in cacao beans. It also found in mate, tea, guarana and cola nuts. In its purified form it is a white microcrystalline powder (MerckIndex). It has a molecular formula of $C_7H_8N_4O_2$ with a molecular weight of 180. 164. Its IUPAC name is 3, 7-Dimethylpurine-2, 6-dione and its synonym names include 3, 7-Dihydro-3, 7-dimethyl-(1H)-purine-2, 6-dione; 3, 7-dimethylxanthine; santheose; theobrominum (Clarke'sAnalysis, 2011). Theobromine has a Log P of -0. 8 and pKa values of <1 and 10. It has solubility in water of 1g in 2000ml so very slightly soluble whereas in boiling water it has a solubility of 1g in 150ml. In 95% ethanol it has a solubility of 1g in 2220ml and is almost insoluble in benzene and ether. Theobromine is soluble in dilute mineral acids and alkali hydroxides (MerckIndex).

1. 7. 2 Absorption

The absorption of theobromine in humans has been investigated in the past. Tarka et al. investigated the kinetics and metabolic disposition of theobromine. In their study they used a salt form of theobromine in solution

(theobromine sodium acetate). Regarding absorption they observed in their six test subjects a mean bioavailability of 96% indicating it is well absorbed from the gastrointestinal tract (Tarka et al., 1983). Another study by Mumford et al. 1996 investigated the differences in absorption rates of methylxanthines when they were given in different forms. The forms were capsules, cola and chocolate, in this study theobromine base was used (Mumford et al., 1996). They found the t_{max} following theobromine when given as a capsule was after around three hours. This was relatively slow when compared to caffeine which had a t_{max} (when given as a capsule) of around 30 minutes. The absorption of theobromine following administration via chocolate was found to be less than the absorption of theobromine when given as a solution (Mumford et al., 1996). The relative bioavailability of theobromine in chocolate was found to be 80% (Arnaud, 2011). Furthermore an interesting finding from Mumford et al. study was that theobromines t_{max} was quicker and C_{max} was greater (C_{max} of 8.05ug per ml after 120 minutes) when theobromine was given as a chocolate compared to when theobromine was given as a capsule (C_{max} of 6.72ug per ml after 150 minutes) (Mumford et al., 1996). This is interesting because when theobromine is given in chocolate it would first have to be released from the chocolate matrix therefore have a greater release time relative to pure theobromine base given in capsule. Mumford et al. has suggested the effect of bile acids as a possible explanation for the chocolate however this has not been proved. Furthermore in relation to the chocolate effect Mumford et al. found caffeine had a slower T_{max} and smaller C_{max} when it was given as a chocolate compared to when it was given as a capsule (Mumford et al.,

1996). This shows the effect of chocolate seems to be different on the absorption of caffeine and theobromine.

1. 7. 3 Distribution

The volume of distribution as found by Tarka et al. was a mean of 0. 68 litres per kilogram (Tarka et al., 1983). Another study done by Lelo et al. investigated the pharmacokinetics of caffeine, theobromine, theophylline and paraxanthine. They found the volume of distribution of theobromine to be around 0. 8 litres per kilogram and the unbound fraction to be around 80% (Lelo et al., 1986). Zhang et al. carried out a study which investigated the binding of theobromine with human serum albumin. From their stern volmer plot they found that dynamic quenching or static quenching was predominant (Zhang et al., 2009). They also found from the stern volmer quenching constant (K_{sv}) was inversely correlated with temperature, at 299K the K_{sv} was $2.17 \times 10^4 \text{ L mol}^{-1}$ and at 309 the k_{sv} was lower at $2.04 \times 10^4 \text{ L mol}^{-1}$, this indicates theobromines quenching of human serum albumin was by complex formation and not by dynamic collision. Furthermore the binding constant was found to be 2.29×10^4 which can be used to calculate the dissociation constant by using the formula $K_a = 1 / K_d$. The K_d was 0. 04mM. And this is the concentration at which half of the proteins binding sites would be occupied.

1. 7. 4 Metabolism

Theobromine is metabolised by CYP P450 into 3-methylxanthine and 7-methylxanthine. These metabolites are produced by the removal of a methyl side group (demethylation). 7-methylxanthine is then metabolised by

xanthine oxidase into 7-methyluric acid. Theobromine is also metabolised into 6-amino-5-(N-methylformylamino)-1-methyluracil and into 3, 7-dimethyluric acid (Tarka et al., 1983). Tarka et al. found in the analysis (by HPLC) of urine that a mean of 79.6% of the dose was recovered in the urine over a 48 hour collection period after a dose of 10mg per Kg of theobromine was administered to the six test subjects. Furthermore the major metabolite found in the urine was 7-methylxanthine and the second major metabolite found in the urine was 3-methylxanthine, these were found in the urine at mean percentages of 33.6% and 19.9% respectively (Tarka et al., 1983). The metabolism of caffeine results in theobromine as demethylation of caffeine results in theobromine (Arnaud, 2011).

1. 7. 5 Elimination

From Lelo et al study they found theobromine to have a half-life of around 8 hours and a total plasma clearance of 1 ml per minute per kilogram (Lelo et al., 1986). It has also been reported theobromine has a half life on average of 7.1 hours after nursing mother's ingested milk chocolate which contained 240mg of theobromine. Furthermore after administration of a 1000mg dose of theobromine 62% of the administered dose was recovered in urine, urine collected over 48 hours. Theobromine is said to be greatly reabsorbed in the renal tubule and theobromine's renal clearance is greatly dependent on urine flow (Arnaud, 2011).

2. AIMS AND OBJECTIVES

2. 1. Aim

The aim of this project is to investigate the solubility of theobromine, in particular its solubility at pHs of the stomach and small intestine in order to gain an insight into how theobromine with its low solubility is well absorbed. And to investigate why theobromine is absorbed faster in a chocolate form compared to a capsule form.

2. 2. Objectives

1) Produce a calibration curve for theobromine dissolved in universal buffer pH 7. 42) Investigate theobromines solubility at acidic pHs of the stomach. 3) Investigate theobromines rate of dissolution at pHs of the stomach and small intestine. 4) Investigate the solubility of theobromine at alkaline pH5) Investigate changes in the absorption spectrum at acidic, neutral and alkaline pHs6) Investigate the effect of bile acids on the solubility of theobromine7) Investigate the effect of various surfactants on the solubility of theobromine

3. METHODS

Standard operating procedures are located in Appendix 1

3. 1. Cleaning UV quartz cuvettes

Glass cuvettes were used throughout this study and due to limited number of cuvettes, they had to be reused. Because the cuvettes were being reused adequate cleaning of cuvettes was important and necessary in order to get accurate reading. Cuvettes after use were first cleaned with distilled water,

then with ethanol and then allowed to dry. If cuvettes were not cleaned properly then the residues left from the previous sample in the cuvette would have affected the absorbance reading of the following sample and so would have distorted the results.

3. 2. UV visible spectrum of theobromine

The maximum absorption wavelength of theobromine was important to determine as throughout the various experiments a spectrophotometer would be used to determine the amount of theobromine in solution. To determine the maximum absorption wavelength 10mg of theobromine was dissolved in 100ml of universal buffer (pH 7. 4) and this was used to do a scan on the spectrophotometer. The maximum absorption wavelength of theobromine when dissolved in universal buffer was found to be 273nm.

3. 3. Calibration curve for theobromine

A calibration curve of theobromine dissolved in universal buffer (pH 7. 4) was to be used in the various experiments to determine the amount of drug dissolved in a particular solution. By making a calibration curve of absorbance versus drug concentration, the graph can be used to determine the amount of drug in solution when the absorbance value is known. Three sets of calibration curves were done. For each set of experiments approximately 10mg (9. 5mg, 10. 2mg and 9. 5mg for first, second and third experiments respectively) was dissolved in 100ml of universal buffer. For example for the first experiment 9. 5mg was dissolved in 100ml of universal buffer. Then a series of dilutions were made which were as following: 1 in 6, 1 in 7, 1 in 8, 1 in 10 and 1 in 20. The spectrophotometer was set to 273nm

wavelength as this was the maximum absorption wavelength determined from a previous experiment. The spectrophotometer was zeroed with cuvette containing just universal buffer. Then each of the previously prepared dilutions were placed in a cuvette and their absorbance's recorded (only absorbance's below 1 were accepted due to accuracy). The absorbance values were plotted on a absorbance versus concentration graph. This was repeated twice to obtain the second and third calibration curves.

3. 4. Investigating theobromines solubility at acidic pHs

Theobromines solubility at the following pHs was investigated; pH 1.06, 1.6, 2.1, 2.5, 3.4 and 4.7- Preparing the various acidic pH solutionsThe solutions were prepared using 1M hydrochloric acid and then bringing the pH (more alkaline) by adding 1M sodium hydroxide drop by drop until the required pH was obtained. The pH value of the solution was monitored using a pH meter whilst the 1M sodium hydroxide was added to reach the required pH.- Creating saturated solutions at the various pH's30ml of each prepared solution was placed into different 50ml beakers. Each beaker was labelled according to its pH. Then using a spatula theobromine was added to each beaker, enough was added to create saturated solutions. All solutions were then stirred with a glass rod for a couple of minutes. The solutions were then left for two days.- Centrifugation of saturated solutionsTo determine how much theobromine had dissolved in the various solutions they first had to be centrifuged to separate the undissolved drug. Using a Gilson pipette 1ml of each pH solution was added to 1ml eppendorfs. Then the six eppendorfs were labelled accordingly and placed in the centrifuge. Care had to be taken

to make sure they were balanced for example if one eppendorf was placed in a hole in the centrifuge the next eppendorf had to be placed in the opposite hole. Then the centrifuge was run for 10 minutes. -Dilutions of centrifuged solutions The solutions once centrifuged need to be diluted in order to get a reading of below 1 on the spectrophotometer. A total of 1 in 100 dilution was needed to get an absorbance value below 1. To get this dilution 100ul of the centrifuged solution was added to another empty eppendorf to which 900ul of solution of the same pH was added to get a 1 in 10 dilution. For example to dilute the pH 1.6 solution which had just been centrifuged, 100ul from the centrifuged eppendorf was added to an empty eppendorf and then 900ul of previously prepared pH 1.6 solution was added, the eppendorf was then closed and inverted at least 3 times to get a good mix. When a sample was taken from the centrifuged eppendorfs tubes care had to be taken when using the 100ul Gilson pipette to stay away from the precipitate and only take sample from the supernatant. This step was repeated again to get a total of a 1 in 100 dilution. -Absorbance reading Once the 1 in 100 dilutions for the various pHs were prepared the absorbance reading of the solutions needed to be taken. The spectrophotometer was set to wavelength 273nm and zeroed with a 1ml quartz cuvette containing water. After the spectrophotometer was ready to use, each diluted sample was placed in a 1ml quartz cuvette, the cuvette was then placed in the spectrophotometer and the absorbance value noted. These set of experiments were repeated two more times to give a total of 3 sets of data.

3. 5. Theobromines rate of dissolution at pH 2 and pH 7

-Preparing the solutions of pH 2 and pH 7
The pH 2 solution was prepared by adding 40ml of 1M hydrochloric acid into a 100ml beaker and then 1M sodium hydroxide was added drop by drop using a pasteur pipette until pH 2 was reached. A pH meter was used to monitor the pH of the solution as sodium hydroxide was added. For pH 7 solution previously prepared universal buffer (pH 7. 4) was used.

-Creating saturated solutions
After the two solutions were prepared 20ml of each was added to vials and labelled accordingly. Then a saturated solution was created by adding theobromine using a spatula, enough was added to produce a saturated solution. These vials were then placed on a roller and samples were taken at regular intervals. The samples were placed on a roller to mimic movement within the stomach.

-Sample taking
At regular intervals (10, 20, 40 and 60minutes) a sample was taken from each vial. This was done by pipetting out 1ml of the sample using a 1000ul gilson pipette and adding to eppendorfs. These eppendorfs were centrifuged for 10 minutes. Care had to be taken to make sure they were balanced for example if one eppendorf was placed in a hole in the centrifuge the next eppendorf had to be placed in the opposite hole.

-Dilutions of centrifuged solutions
Once the samples were centrifuged dilutions were carried out in order to obtain a reading of below 1 on the spectrophotometer. To get this dilution 100ul of the centrifuged solution was added to another empty eppendorf to which 900ul of the same pH solution was added (i. e. centrifuged pH 2 solution was diluted with pH 2 solution and centrifuged pH 7 solution was diluted with pH 7 solution). This would give a 1 in 10 dilution. When a sample was taken from the centrifuged eppendorfs

tubes care had to be taken when using the 100ul Gilson pipette to stay away from the precipitate and only take sample from the supernatant. This step was repeated again to get a total of a 1 in 100 dilution. Once a 1 in 100 dilution was achieved the sample was placed in a 1ml UV quartz cuvette and placed in a spectrophotometer set at 273nm. The absorbance value was then recorded. These set of experiments were repeated two more times to give a total of 3 sets of data.

3. 6. Solubility of theobromine in sodium hydroxide

-Preparing various sodium hydroxide concentrations
First the various sodium hydroxide concentrations had to be prepared. 1M sodium hydroxide solution and distilled water were used to prepare the various solutions. They were added at different ratios depending on the concentration needed, the table below shows how much of each solution was added to make 20ml solutions of the various concentrations. Sodium hydroxide concentration Volumes added

Sodium hydroxide concentration	Volumes added
1M	20ml of 1M sodium hydroxide 0ml of distilled water
0.5M	10ml of 1M sodium hydroxide 10ml of distilled water
0.2M	4ml of 1M sodium hydroxide 16ml of distilled water
0.1M	2 ml of 1M sodium hydroxide 18ml of distilled water
0.01M	0.2ml of 1M sodium hydroxide 19.8ml of distilled water

-Creating saturated solutions
10ml of each previously prepared sodium hydroxide solutions (1M, 0.5M, 0.2M 0.1M and 0.01M) were added to separate 20ml scintillation vials and labelled accordingly. Then using a spatula theobromine was added to each vial, enough was added to create saturated solutions. Each vial was then closed and shaken vigorously for a couple of minutes.

-Centrifugation
Centrifugation had to be carried out next to

separate the undissolved drug from the dissolved drug. Using a 1ml Gilson pipette 1ml of each sample was added to empty eppendorf tubes and labelled accordingly. The eppendorf tubes were then placed into the centrifuge. Care had to be taken to make sure they were balanced in the centrifuge, for example if one eppendorf was placed in a hole in the centrifuge the next eppendorf had to be placed in the opposite hole. The centrifuge was run for 10 minutes. -Dilutions Once the centrifuge had been complete samples from each eppendorf tube had to be taken and diluted in order to get an absorbance reading on the spectrophotometer of below 1. When a sample was taken from the centrifuged eppendorfs tubes care had to be taken when using the 100ul Gilson pipette to stay away from the precipitate and only take sample from the supernatant. Dilutions were done in steps of 1 in 10 dilutions until an absorbance of below 1 was achieved. Dilutions for each concentration of sodium hydroxide were done using the same concentration of sodium hydroxide, for example for a dilution of the 0.5M sample 100ul of the sample was diluted with 900ul of 0.5M sodium hydroxide solution. Absorbance readings were done using 1ml UV quartz cuvettes on spectrophotometer set at 273nm wavelength. The spectrophotometer was zeroed against 1ml UV quartz cuvettes containing universal buffer pH 7.4. These set of experiments were repeated two more times to give a total of 3 sets of data.

3. 7. Effect of pH on the maximum absorption wavelength of theobromine

A spectrophotometer was used to determine the maximum absorption wavelength of theobromine in solutions of various pHs (pH 7.4, 1.5 and 13).

5).-Maximum absorption wavelength at pH 7. 4For determining the maximum absorption wavelength for theobromine at neutral pH universal buffer (pH7. 4) was used. Approximately 10mg of theobromine was dissolved in 100ml of universal buffer in a 100ml conical flask. The conical flask containing the theobromine and 100ml of universal buffer was shaken for a minute and then left in the sonicator until all the drug had dissolved. Then a 1ml UV quartz cuvette containing just universal buffer was used to calibrate the spectrophotometer. After it was calibrated the cuvettes were filled with the solution containing theobromine dissolved in universal buffer. A 1 in 10 dilution was needed as the absorption spectra was off scale so 100ul of the previously prepared theobromine dissolved in universal buffer was added to the cuvette along with 900ul of just universal buffer. Then a scan was ran which was not of scale and the results revealed the maximum absorption wavelength of theobromine at pH 7. 4. This step was repeated for theobromine dissolved in pH 1. 5 and 13. 5 solutions.-Making pH 1. 5 and pH 13. 5 solutions. The solutions of pH's 1. 5 and 13. 5 were prepared using 1M hydrochloric acid and 1M sodium hydroxide. First the pH meter had to be calibrated. To make the pH 1. 5 solution 120ml of 1M hydrochloric acid was added to a 200ml beaker and then 1M sodium hydroxide was added in a drop wise manner using a pasteur pipette until the pH reached pH1. 5. To make the pH 13. 5 solution 120ml of 1M sodium hydroxide was first added to a 200ml beaker and then 1M hydrochloric acid added in a drop wise manner using a pasteur pipette until pH 13. 5 was reached.-Maximum absorption wavelength at pH 1. 5 and pH 13. 5The steps followed to determine the maximum absorption wavelength for theobromine in universal buffer as

described earlier were followed to determine maximum absorption wavelength of theobromine at pH 1.5 and pH 13.5. The steps involved adding 100ml of pH 1.5 solution to 100ml conical flask which was then labelled accordingly. Then 10mg of theobromine was added to the conical flask. The conical flask was then left in a sonicator and shaken regularly until all the theobromine had dissolved. Once they had dissolved the spectrophotometer was calibrated using a 1ml UV quartz cuvette containing previously prepared pH 1.5 solution. After it was calibrated a 1ml UV quartz cuvette was filled with the solution containing theobromine dissolved in pH 1.5 solution. A 1 in 10 dilution was needed as the absorption spectra was off scale so 100ul of the previously prepared theobromine dissolved in pH 1.5 solution was added to the cuvette along with 900ul of just pH 1.5 solution. Then a scan was ran which was within scale. This step was repeated for theobromine dissolved in pH 13.5 solution.

3. 8. Effect of sodium deoxycholate on the solubility of theobromine.

A stock solution of 10mM sodium deoxycholate was prepared by adding 2.073g of sodium deoxycholate into a 500ml conical flask add then made up to volume with universal buffer. Universal buffer was added slowly to avoid the formation of foam. Once stock solution was prepared various dilutions were prepared in 20ml vials. The following strengths were prepared; 1, 1.5, 2, 3, 4, 6, 8 and 10mM. Then using a spatula theobromine was added to each vial, enough was added to create saturated solutions. These were then shaken for up to a minute and then left to stand for 30 minutes. After this a sample was taken (approx. 1ml) from each vial and placed in to eppendorfs to be

centrifuged for 10 minutes. Care had to be taken to make sure they were balanced for example if one eppendorf was placed in a hole in the centrifuge the next eppendorf had to be placed in the opposite hole. Once centrifuged the samples were diluted 1 in 100 to get an absorbance reading of below 1. Each sample was placed in a 1ml UV quartz cuvette and then placed into the spectrophotometer (set at 273nm and zeroed against a 1ml UV quartz cuvette containing just universal buffer) to record its absorbance value. This experiment was repeated two more times to give a total of 3 sets of data.

3. 9. Effect of surfactants CHAPS and SDS on the solubility of theobromine.

-Preparing various concentrations of surfactants CHAPS and SDS Initially stock solutions of both surfactants were prepared. For CHAPS a stock solution of concentration 15mM was prepared. This was done by adding 0.922 grams of CHAPS to a 100ml and then making up to volume by adding universal buffer (pH 7.4). Care had to be taken when adding universal buffer in order to minimise the formation of foam. For SDS a stock solution of concentration 20mM was prepared by adding 0.576 grams of SDS to a 100ml conical flask and then making up to volume with universal buffer. Once the stock solution were prepared various dilution were done using universal buffer to create various solutions of various concentrations. The following concentrations were prepared; For CHAPS - 1.5, 3, 4.5, 6, 7.5, 9, 10.5 and 15mM For SDS - 2, 4, 6, 8, 10, 15 and 20mM 10ml of each was added to 20ml vials and labelled accordingly. -Creating saturated solution After the various concentrations were prepared and added to vials and labelled accordingly. Theobromine was added, using a spatula, to each

vial. Enough theobromine was added to create saturated solutions. Once saturated solutions were created the lids were placed on the vials and then the vials shaken vigorously for a two to three minutes. After this the vials were left for 30 minutes during which the vials were shaken every couple of minutes.-CentrifugationAfter approximately 30 minutes 1000ul was taken from each vial using a 1000ul Gilson pipette and added to empty eppendorf tubes which were labelled accordingly. These eppendorfs were then added to a centrifuge. Care had to be taken to make sure they were balanced in the centrifuge, for example if one eppendorf was placed in a hole in the centrifuge the next eppendorf had to be placed in the opposite hole. The centrifuge was ran for 10 minutes.-Dilutions of centrifuged solutionsThe solutions once centrifuged need to be diluted in order to get a reading of below 1 on the spectrophotometer. A total of a 1 in 100 dilution was needed to get an absorbance value below 1. To get this dilution 100ul of the centrifuged solution was added to another empty eppendorf to which 900ul of solution of the same surfactant concentration was added to get a 1 in 10 dilution. For example to dilute the 20mM SDS solution which had just been centrifuged, 100ul from the centrifuged eppendorf was added to an empty eppendorf and then 900ul of previously prepared 20mM SDS solution was added, the eppendorf was then closed and inverted at least 3 times to get a good mix. When a sample was taken from the centrifuged eppendorfs tubes care had to be taken when using the 100ul Gilson pipette to stay away from the precipitate and only take sample from the supernatant. This step was repeated again to get a total of a 1 in 100 dilution.-Absorbance readingOnce the 1 in 100 dilutions for the various surfactant concentrations were

prepared the absorbance reading of the solutions needed to be taken. The spectrophotometer was set to wavelength 273nm and zeroed against universal buffer. After the spectrophotometer was ready to use, each diluted sample was placed