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Colorimetric determination of acetaminophen degradation in blood samples stored at 24ºC and 40ºC

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ABSTRACT

Acetaminophen (APAP) overdose fatalities have decreased slightly over the last few years but still remain ranked in the top 10 by the American Association of Poison Control. Due to APAPs chemical composition it can undergo various possible degradation pathways. Previous studies utilized thermal stressing and measured the rate of degradation by High Performance Liquid Chromatography, however the degradation of APAP within biological samples has not been well established. In this study the degradation of APAP was studied in bovine blood at 24ºC and 40ºC over a four week period. When comparing the initial and final concentration of APAP in the blood samples, the final measurement was 14.41% higher for the 24^0C and 11.79% higher for the 40^0C samples. However, results obtained were not statistically significant enough to conclude that the change was due to the storage of the blood samples at 24ºC and 40ºC. There was a slight increase in the first measurement recorded for both the 24°C and 40ºC samples which could suggest that there was a loss of volume upon storage.

Keywords: *Acetaminophen, Overdose, Blood, Colorimetric*

INTRODUCTION

Acetaminophen or N-acetyl-para-aminophenol (APAP) is a commonly used analgesic and antipyretic drug that is prescribed as an alternative long-term treatment to aspirin in order to decrease the risk of gastrointestinal bleeding (Diamond 2000). The prescribed single dose of APAP for adults is 500-1000 mg, and may be repeated every 4 to 6 hours with a maximum combined dosage of 4000 mg in 24 hours (Tylenol 2016). APAP is expediently available over the counter without a prescription, which has resulted in the drug becoming misused in the United States (Mowry et al. 2016). APAP overdose is defined as the ingestion of a minimum concentration of 140 mg/kg at which toxicity is initiated (Kolambabe and Soysa, 2010), dosages greater than 350 mg/kg result in hepatic damage, and in severe cases hepatic failure and death (Larson 2005). APAP is metabolically activated by the cytochrome P450 by a two electron oxidation process to form a reactive metabolite N-acetyl-p-benzo-quione imine (NAPQI). At a toxic dose NAPQI binds to cysteine groups causing centrilobular hepatic necrosis (Davern 2006).

APAP fatalities have decreased slightly over the past 10 years, however APAP still ranks yearly among the top 10 largest number of fatalities out of the cases studied by the American Association of Poison Control Centers (AAPCC) (Mowry et al. 2016). Figures from the 2015 Annual Report of the AAPC indicated that out of 1256 cases studied 143 APAP (alone) and 135 APAP (combination) deaths occurred in 2015 (Mowry et al. 2016), compared to 130 (alone) and 240 (combination) deaths in 2010 out of 1366 cases studied (Bronstein, et al., 2011) and 47 (alone) and 92 (combination) deaths in 2005 out of 416 studied (Lai, et al., 2006). Resulting in 22.13% in 2015, 27.09% in 2010 and 33.41% in 2005 of fatalities in the cases studied are due to APAP. Other research states that APAP overdose was the leading cause of acute liver failure between 1998 and 2003 (Larson 2005).

Numerous cases of APAP overdose are accidental or unintentional due to a rapid increase in APAP/ opioid combination medications manufactured in recent years (Bunchorntavakul and Reddy 2013). As a result, the FDA released compliance statements aimed at APAP manufacturers to include a warning to patients on the packaging of any APAP combination medications and to distinctly state the concentration of APAP in the medication and the danger associated with using the drug in combination with another source of APAP (U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) 2017). Currently there are 48 prescription and non-prescription APAP containing drugs available. Some commonly known prescription drugs containing APAP are: Endocet®, Fioricet®, HycotabHydrocet®, Hydrocodone, Bitartrate, Lortab®, Percocet®, Phenaphen®, Sedapap®, Tapanol®, Tylenol® with Codeine, Tylox®, Ultracet®, Vicodin®, Zydone® (KnowYourDose.org, 2015).

Due to the fact that APAP is not considered as a high-importance abuse drug, such as heroin or cocaine, information regarding the stability of APAP within biological samples over a period of time is not yet well established. It is suspected that due to APAP's chemical structure it is subjected to chemical degradation by a variety of pathways. Previous studies utilized conventional thermal stressing and measured the rate of decomposition by high-performance liquid chromatography (HPLC) and provided a predicted rate of decomposition over a five-year period (Gilpin and Zhou, 2004). This research

was not conducted within biological samples.

This is of high importance as incorrect analysis and the unreliability of the analytical results can severely affect the development and outcome of forensic services. The standard procedure of drug testing is to establish a chain of custody and analyze samples using an accurate and reliable technological procedure (James 2012). If the reliability of the evidence is questionable, then the evidence is inadmissible in a court of law.

The goal of my study is to understand the effect that temperature has on the stability of APAP contained in the samples. Therefore, this study aims to determine the rate of degradation of APAP within the blood using a colorimetric assay. This method is based on the formation of 2-nitro-5-acetamidophenol produced through the reaction of APAP with nitrous acid which produces a yellow colored compound. The color produced is measured at a wavelength of 430nm and is proportional to the concentration of APAP in the sample (Hale and Polkis, 1983).

MATERIALS AND METHODS

Method altered from Hale and Polkis (1983).

Apparatus

A Spectrotroninc Genesys 2 UV spectrometer was used for all of the colourimetric determinations. A Isotemp 215 water bath was used.

Reagents

4-Acetamindophenol and sodium hydroxide obtained from Acros Organics was used to make 2g/L and 8M solutions respectively. 3% Trichloroacetic acid (w/v) was obtained from Sigma Aldrich, 0.07M sodium nitrite was obtained from Fisher Scientific, and 3.8% sodium citrate (w/v) was obtained from Merck. All chemicals used were supplied by McPherson College.

Standards

200mg of APAP was weighed out and dissolved in 100mL of deionized water to obtain a 2g/L stock APAP solution. A series of dilutions were then made up at 1.5g/L, 1g/L, 0.5g/L and 0.25g/L solutions.

Calibration Curve

200uL aliquots of the APAP solutions were added to 1mL of fresh citrated bovine blood which was obtained through Carolina Biologicals, to obtain 50, 100, 200, 300 and 400 mg/L samples used to construct the calibration curve.

Storage

Blood samples spiked with APAP were stored in 20mL centrifuge tubes, in an incubator at 24°C and 40°C.

Serum

25mL of fresh citrated bovine blood was spiked with 5mL of 1.5g/L APAP stock solution. 1mL aliquots of spiked blood as well as non-spiked blood was then placed in 1mL micro centrifuge tubes for initial testing and the remaining blood was stored. 1mL blood aliquots were centrifuged at 10 000g for 10min initially as well as after storage time in order to obtain serum.

Sodium Citrate

3.8g of sodium citrate was added to 100mL of deionized water to obtain at 3.8% (w/v) solution. 0.1mL of solution was added to 0.9mL of deionized water to form a 1:9 ratio contained in blood samples. A sodium citrate APAP blank sample was analyzed with each run.

Colorimetric determination

3mL of 3% Trichloroacetic acid solution was added to a Pyrex No. 9826 13x10cm test tube containing 0.3mL of serum as well as a test tube containing 0.3mL of sodium citrate/ APAP sample. The solutions were then vortex mixed for approximately five seconds and centrifuged at 3500g for five minutes.

2mL of the supernatant was transferred to clean test tubes and 0.5mL of 0.07M sodium nitrite solution was added. The solutions were vortex mixed again for approximately five seconds and placed in a water bath set at 37°C for 10 minutes.

After this period two drops of 8M sodium hydroxide was added which initiated the color change and the solutions were vortexed for the last time for 5 seconds. The absorbance of the colored solution was then measured at a wavelength of 430nm about a minute after the sodium hydroxide addition.

Statistical Analysis

A paired-samples t-test was conducted to compare the degradation of APAP in samples stored at 24°C and 40°C

RESULTS

The purpose of this experiment was to measure the concentration of APAP in blood samples stored at 240C and 40°C, in order to detect a change or degradation in the concentration.

Calibration Curve

An APAP serum calibration curve was constructed for the method by using concentrations between 50mg/L and 300mg/L. Figure 1 represents the calibration curve for APAP in serum, each point is representative of the mean of three values obtained. The trend-line equation obtained was: $y=0.0005x+0.0263$ with an $R^2=0.9979$. Values obtained were corrected for a serum blank.

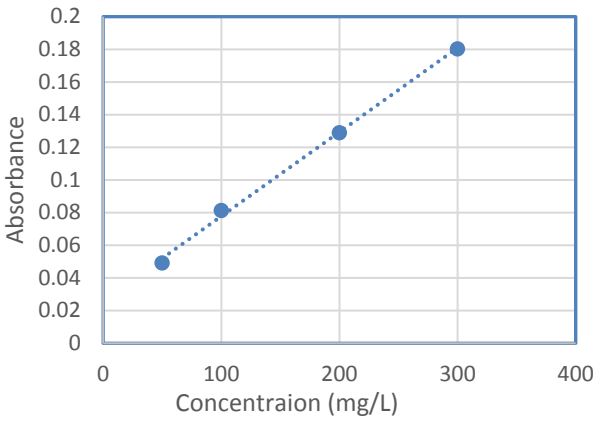

Figure 1. Calibration curve constructed in bovine serum with varying APAP concentrations.

Blood Samples

The absorbance of the spiked blood samples was measured weekly over four weeks and corrected for a serum blank as well as sodium citrate sample, which was subjected to the same experimental conditions.

Using the trend-line obtained from the calibration curve, the absorbance obtained from the samples was converted to the concentration of APAP in the serum. The concentrations obtained for each measurement are shown in Figure 2. The total amount of blood samples analyzed was 24. APAP concentrations increased over 592 for both the 24°C and 40°C by 30.67mg/L and 30.67mg/L respectively.

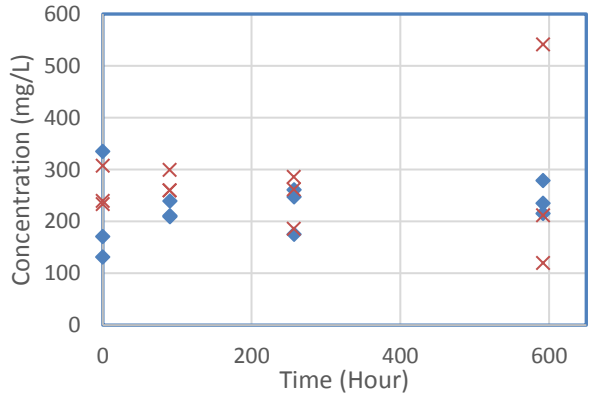

Figure 2. Concentration of 2-nitro-4-Acetamidophenol in 24 samples stored at 24°C (represented as diamonds) and 40°C (represented as crosses) over a period of four weeks.

The concentration of APAP measured in samples is also represented as a percentage of the initial APAP concentration shown in Figure 3. There was an overall percentage increase of 14.41% for the 24°C series and 11.79% for the 40°C series.

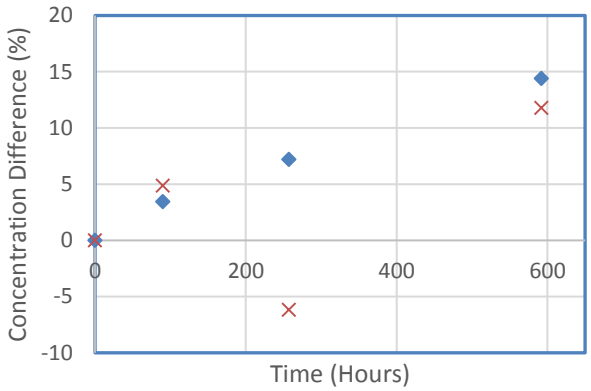

Figure 3. Change in APAP concentration represented as a percentage of the initial concentration measured. Samples stored at 24°C are represented as diamonds and 40°C represented as crosses.

DISCUSSION

The method used in this study is a very cost effective and easily accessible method to analyze the concentration of APAP in serum, when compared to the cost and availability of analysis done by HPLC.

Previous studies, that utilized thermal stressing to degrade APAP over a five-year period, provided a relative APAP degradation percentage at 35°C and pH 6 and 8 (Gilpin and Zhou, 2004). These degradation rates were used as a guide for the current study since blood pH is around 7.4. The aim of our work was to determine if Gilpin and Zhou's (2004) degradation rates apply in a biological matrix at 24°C and 40°C.

A paired-samples t-test was conducted for the current study, to compare the degradation of APAP in samples stored at 24°C and 40°C over a period of 592 hours. There was not a significant difference in degradation for samples stored at 24°C and 40°C respectively, with t=-2.81 and P=0.068. I am therefore unable to conclude that the change in concentration observed between the 24°C and 40°C series was due to their storage conditions.

A previous study focused on the postmortem pharmacokinetics of APAP and was conducted on New Zealand white rabbits (Gomez et al. 1995). This study saw an overall blood APAP concentration increase and concluded that post-mortem drug concentrations do not reflect pre-mortem values. Which is consistent with the overall percentage increase observed in the current study.

A possible explanation for the slight increase in concentration observed in my study for both the samples stored at 24°C and 40°C, would be a decrease in sample volume. This would be misrepresented as a concentration increase since concentration is a measure of mass per unit volume (mg/L). However, samples were stored in capped

20mL centrifuge tubes.

For future studies I would increase the sample size as n=12 for each temperature group was not a statistically sufficient number of samples (P>0.05). However, a sample size of n=33 for each temperature group would provide a power of 0.8 and detect a 10mg/L change in concentration.

This analytical method is very cost effective and would therefore allow for the repetition of this study by increasing storage temperature, storage time and sample size which would allow for a better understanding of degradation of APAP in bovine blood.

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