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EFFECTS OF THEOPHYLLINE ON CYP1A2-MEDIATED CAFFEINE BIOTRANSFORMATION IN HUMAN LIVER MICROSOMES

*Farhana Ali[1], Zeyad Alehaideb[2] and Francis CP Law[1]
[1]Department of Biological Sciences, Simon Fraser University, British Columbia V5A 1S6, Canada
[2]Department of Medical Genomics, King Abdullah International Medical Research Center / King Saud Bin Abdulaziz University for Health Sciences, PO. Box 22490 Riyadh 11426, Saudi Arabia

ABSTRACT

Caffeine (1,3,7-trimethylxanthine) is a major active component of many foods, beverages, dietary supplements and medications. Theophylline (1, 3-dimethylxanthine) is a drug used to dilate and stimulate the respiratory tract. The aims of this study were: to determine the maximum velocity rate of cytochrome (CYP) 1A2-mediated caffeine biotransformation using NADPH-fortified human liver microsomes (HLMs), to determine *in vitro* inhibitory potency (IC_{50}) and inhibition constant (K_i) of theophylline on caffeine biotransformation, and to provide a mechanistic explanation for caffeine/theophylline interaction. To this end, caffeine was incubated with NADPH-fortified HLMs in the presence and absence of theophylline. The K_M and V_{max} of caffeine 3-*N*-demethylation were determined without adding any theophylline to the incubation; they were 0.66 ± 0.06 mM caffeine concentration and 106.3 ± 3.4 ng of paraxanthine/hour/mg protein, respectively. The IC_{50} and K_i were determined after adding theophylline to the incubation; they were 75.8 ± 5.2 µM and 0.41 ± 0.03µM of theophylline concentrations, respectively. Our study also showed theophylline probably acted as a competitive inhibitor of caffeine metabolism. In view of the popularity of caffeinated beverages and adverse health effects of caffeine, care must be exercised when caffeine and theophylline are consumed together.

Keywords: Caffeine metabolism, theophylline, enzyme kinetics, cytochrome P450 inhibitors.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a xanthine alkaloid and a stimulant of the human brain. Caffeine is usually used to restore mental alertness. It is also used as remedies for cold and headache (Graham, 2001; Solinas *et al*., 2002). Caffeine is principally biotransformed by the hepatic cytochrome (CYP) P450 oxidases system to three dimethylxanthine metabolites: paraxanthine (84%), theobromine (12%) and theophylline (4%) (Fig. 1), each with some biological affects (Krul and Hageman, 1998) and may be further metabolized or excreted into the urine. Indeed, Ha *et al*. (1996), Petersen *et al*. (2006), and Kot and Daniel (2008) have shown that caffeine is primarily demethylated by hepatic CYP1A2 isoenzyme to paraxanthine although other CYP450 isozymes also participate in its biotransformation. Based on the 3-*N*-demethylation (Ha *et al*., 1996; Petersen *et al*., 2006) and/or the 1-*N*-demethylation (Kot and Daniel, 2008) metabolic pathways, caffeine is often used as a probe substrate to determine the activity of hepatic CYP1A2 isozyme.

In contrast, theophylline (1, 3-dimethylxanthine) is a bronchodilator and respiratory tract stimulant; it is an effective agent for the treatment of acute and chronic asthma, Cheyne-Stokes respirations, and apnea/ bradycardia episodes in the newborn. It is also used as an adjunct in the treatment of congestive heart failure and acute pulmonary edema (Hendeles and Weinberger, 1983). The therapeutic range of theophylline is about10-20 mg/l of blood. Theophylline is metabolized extensively in the liver, up to 90% in the adults. It undergoes CYP1A2-mediated *N*-demethylation and 8-hydroxylation to 1-methylxanthine, 3-methylxanthine and 1,3-dimethyluric acid. It is also catalyzed by the CYP2E1 isozyme (Haley, 2008).

The objectives of our study were: (1) to determine the maximum velocity rate of caffeine 3-*N*-demethylation using NADPH-fortified human liver microsomes (HLMs), (2) to determine the *in vitro* IC_{50} and K_i of theophylline on caffeine metabolism, and (3) to provide a mechanistic explanation for caffeine/theophylline interaction.

*Corresponding author e-mail: saraa@sfu.ca

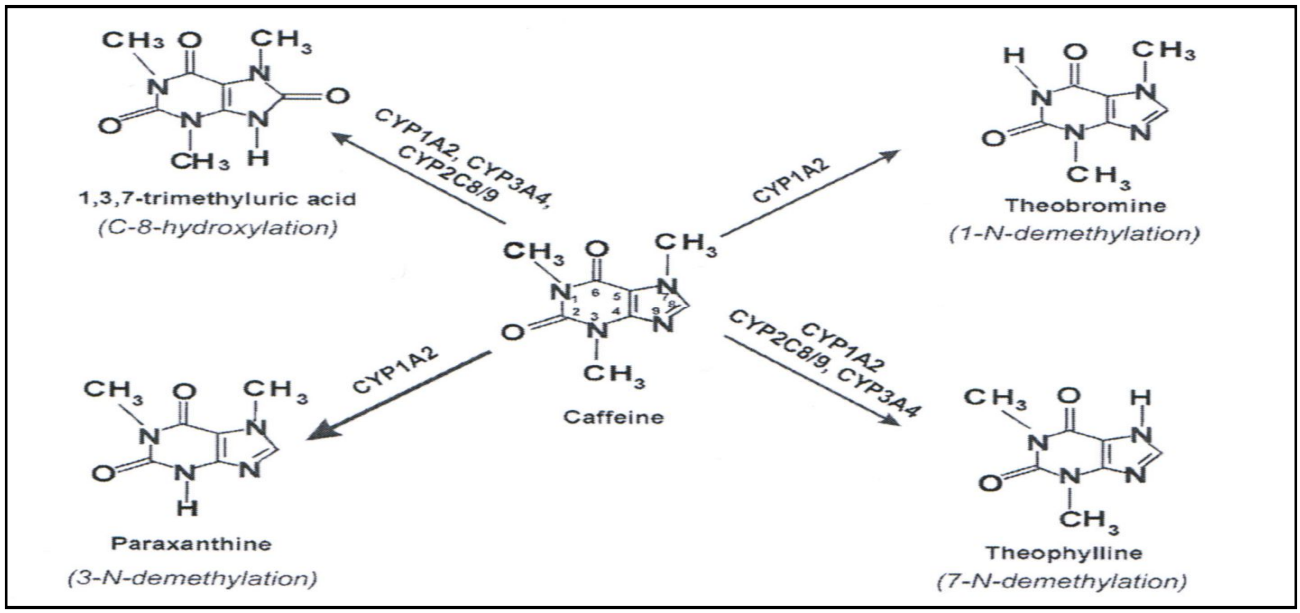

Fig. 1. Caffeine biotransformation by human liver P450 cytochrome isozymes. Paraxanthine is the major metabolite of caffeine 3-*N*-demethylation. (Reprinted from Kot and Daniel (2008) with permission from Elsevier).

MATERIALS AND METHODS

Chemicals and Biological Materials

Caffeine, paraxanthine, theophylline, adenine (6-aminopurine), ethanol, 7-[β-hydroxyethyl]theophylline, and β-nicotinamide adenine dinucleotide phosphate (β-NADPH) were obtained from Sigma-Aldrich (St. Louis, MO). Acetic acid, sodium phosphate monobasic/dibasic, hydrochloric acid (HCl), and ammonium sulphate were obtained from Anachemia (Norman Lachine, QC). Methanol, dichloromethane, and isopropanol were obtained from Caledon (Georgetown, ON). Ethyl acetate was purchased from Fisher Scientific (Hampton, NH). Pooled HLMs were purchased from Becton Dickinson (NYC, NY); the protein concentration was 10 mg/ml. Double distilled water was produced using a Millipore (Burlington, MA) system with a minimum measured resistivity of 16.0 MΩ at 25˚C.

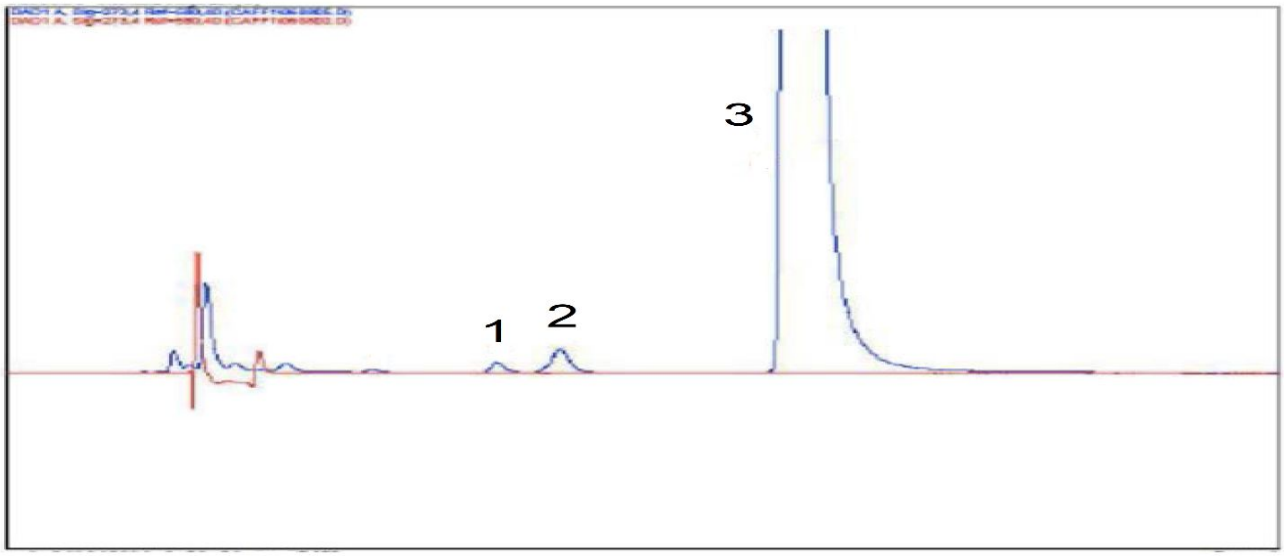

Fig. 2. A typical HPLC chromatogram shows the separation of caffeine (3) and paraxanthine (1). 7-[β-hydroxyethyl] theophylline (2) was used as an internal standard in caffeine biotransformation assay.

In vitro Biotransformation of Caffeine

In vitro caffeine metabolism was carried out in incubation containing HLMs according to Ha *et al*. (1996) with modification. A typical incubation mixture consisted of caffeine (0.128-32.78 mM), pH 7.4 sodium phosphate buffer, and 1.12mM of NADPH in a final volume of 512.5 µl. After a 2 min pre-incubation period, the reaction was started with the addition of 0.25 mg HLMs. After further 2 hr incubation at 37°C, the reaction was stopped by adding 50µl 2M HCl. The internal standard (7-[β-hydroxyethyl]theophylline), 0.5g ammonium sulfate, and 7ml dichloromethane: isopropanol (80:20 v/v) were then added. The incubation mixture was shaken for 10 min and centrifuged for 5 min at 3500 rpm to separate the layers. The organic phase was transferred to a test tube and evaporated to dryness. The residue was re-dissolved in 150 µl of mobile phase. About 120µl of the solution was injected into the HPLC to analyze for paraxanthine.

High Performance Liquid Chromatography

The organic extracts from the incubation were analyzed using an Agilent 1090 high performance liquid chromatography (HPLC) (Palo Alto, CA) equipped with an Agilent ZORBAX Eclipse Plus-C18 95 Å reverse-phase column (4.6 x 250 mm i.d., 5µm), a vacuum degasser, binary pump, auto sampler, and an ultraviolent detector (UVD). The HPLC system was controlled by HP1090 series Chemstation® software.

Caffeine and paraxanthine were separated on an HPLC using isocratic elution (Bispo *et al*. 2002) (Fig. 2). The mobile phase consisted of a solution of methanol: water: acetic acid (75:20:5 v:v:v).The flow rate of the mobile phase was 0.7 ml/min. The column was kept at room temperature and the xanthines were detected at 273 nm. Caffeine and its metabolites were identified by spiking the incubation extracts with pure reference standards. The amount of paraxanthine formed in the incubation was quantified using the internal standard method. Thus, 7-[β-hydroxyethyl] theophylline was used as the internal standard for incubations without theophylline and adenine was used as the internal standard for incubations with added theophylline. Linearity of the calibration curve was established using the B.E.N. Software (DIN 32645) developed by the Institute of Legal Medicine and Traffic Medicine, Germany. The coefficient of linearity for paraxanthine was >0.99. The LOD and LOQ were 0.9 ng/ml and 3 ng/ml, respectively.

K_m and V_{max} Determination

Caffeine biotransformation rate was expressed as ng paraxanthine formed/hr/mg microsomal protein and was plotted against caffeine concentrations in the incubation. The plot showed typical Michaelis-Menten kinetics. The apparent affinity constant (K_m) and maximal rate (V_{max}) were determined directly from the plot.

IC_{50} and K_i Determination

To determine the IC_{50} value, caffeine (864 µM) was incubated with different concentrations of theophylline. The K_M, V_{max}, and IC_{50} values were used to help choose the concentrations of caffeine and theophylline used in the 3x3 design study of Kakkar *et al*. (2000). Thus, the K_i study was conducted using 432, 554 and 864 µM caffeine concentrations, and 977.60, 97.76 and 9.77µM theophylline concentrations.

Data Analysis

GraphPad Prism (San Diego, CA) version 5.0 software was used to determine the K_M, V_{max}, IC_{50} and K_i values by fitting the *in vitro* data to an enzyme kinetic model. Thus, Michaelis-Menten kinetics was assumed in determining the K_M and V_{max} of caffeine metabolism. The IC_{50} value was obtained by analyzing the data with non-linear regression analysis. The K_i and caffeine/theophylline interaction mechanism were determined by fitting multiple datasets simultaneously to the mixed-mode inhibition model of Copeland (2005). Thus, the interaction mechanism would depend on the alpha factor of the mixed-mode inhibition model. The inhibition mechanism would be non competitive if the alpha value were equal to 1. The inhibition mechanism would be uncompetitive if the alpha value were very small. The inhibition mechanism would be competitive if the alpha value were very large.

Statistical Analysis

Results of our study were reported as mean ± SD of three independent experiments.

RESULTS AND DISCUSSION

Figure 3 shows that *in vitro* caffeine biotransformation by HLMs follow the Michaelis-Menten kinetics. The K_M and V_{max} of caffeine 3-N-demethylation were 0.66 ± 0.06 mM caffeine and 106.3 ± 3.4ng paraxanthine/hour/mg proteins, respectively. The K_M and V_{max} are very close to the K_M and V_{max} values reported in the literature. For example, a 0.5 mM K_M of caffeine metabolism was reported by Bloomer *et al*. (1995) and Campbell *et al*. (1987). Other K_M values reported were 0.8 mM (Fuhr *et al*., 1992) and 1.0 mM (Valero *et al*., 1990). The V_{max} of paraxanthine formation in the present study, 106.3 ± 3.4ng paraxanthine/hour/mg, was converted to the same units reported in the literature; it was equal to 9.81 pmol/min/mg. Our V_{max} is slightly less than the 12 pmol/min/mg V_{max} reported by Bloomer *et al*. (1995). In contrast, Butler *et al*. (1989) have reported the

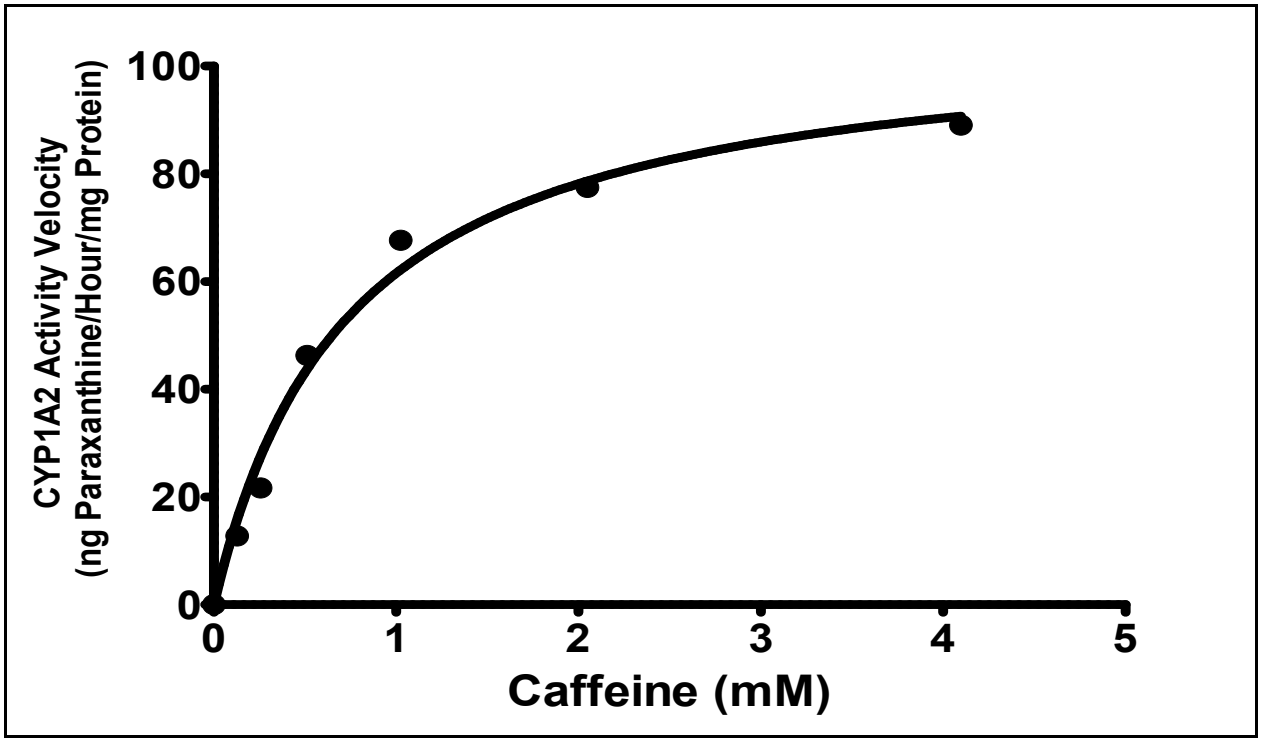

Fig. 3. Effect of caffeine concentration on the formation rate of paraxanthine. K_M and V_{max} were obtained by non-linear regression analysis of the Michaelis-Menten kinetic curve.

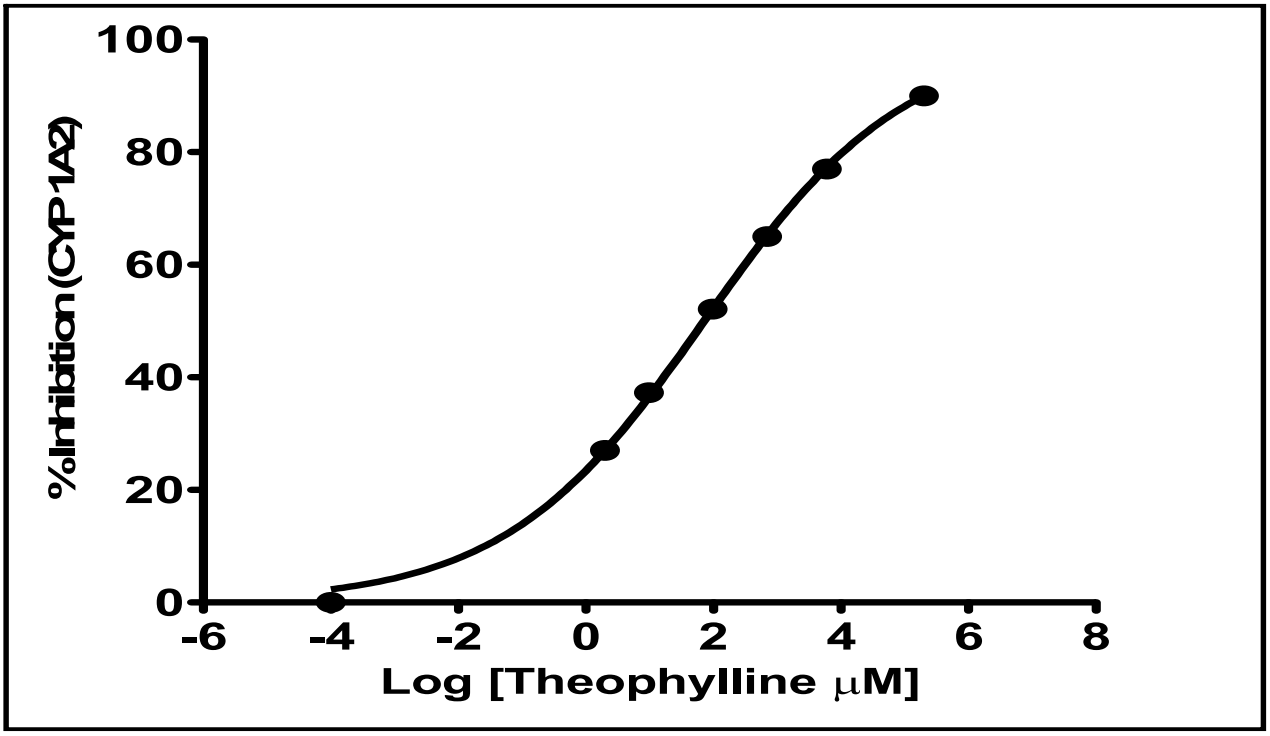

Fig. 4. Effect of theophylline concentration on caffeine metabolism inhibition. IC_{50} was obtained from non-linear regression analysis of the sigmoidal curve.

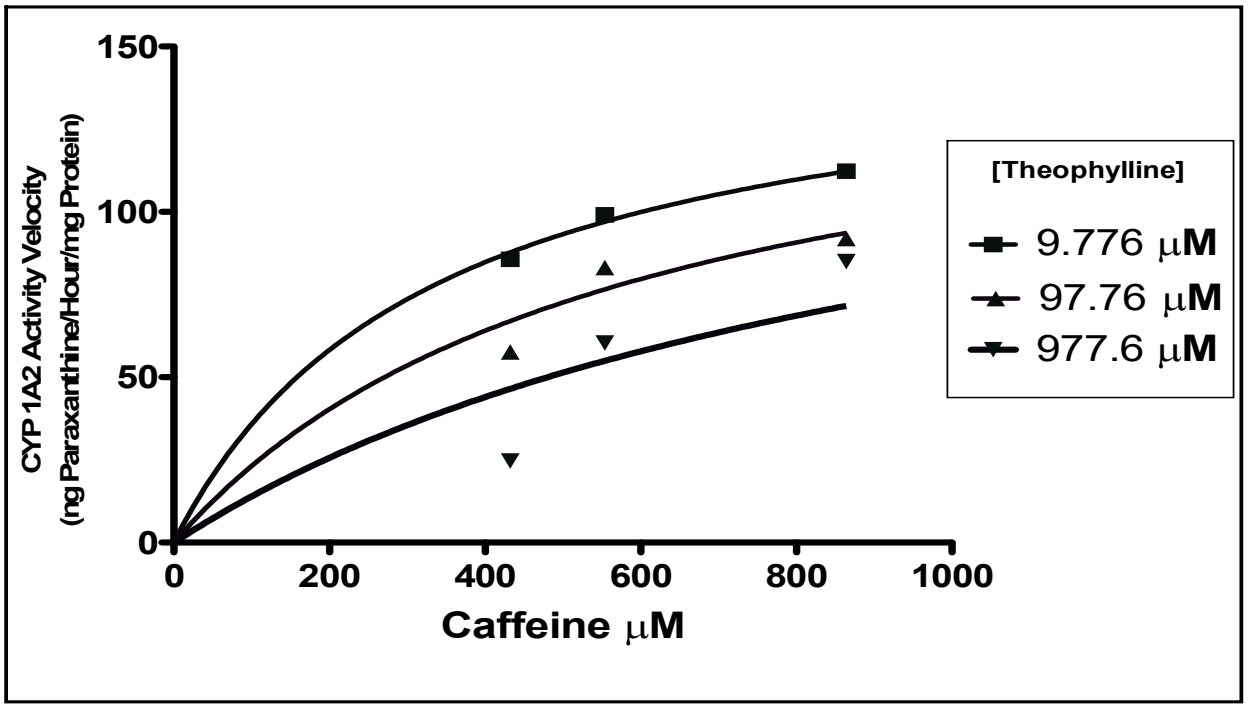

Fig. 5. Effect of theophylline concentration on the rate of paraxanthine formation. K_i was obtained from non-linear regression analyses of these three curves.

lower range of V_{max} value to be 8 pmol/min/mg whereas Grant *et al*. (1987) report the lower range value to be 46 pmol/min/mg.

Figure 4 shows the effects of increasing theophylline concentrations in the incubation on caffeine metabolism inhibition. Non-linear regression analysis was conducted on the log theophylline concentrations (μM) vs. enzyme activity inhibition plot. The IC_{50} of caffeine 3-N-demethylation was 75.8 ± 5.2 μM of theophylline.

Figure 5 shows the effect of an increasing theophylline concentration on the rate of paraxanthine formation. The K_i was determined to be 0.41 ± 0.03 μM of theophylline. The alpha value of the inhibition study was about 7e+12. Therefore, theophylline most likely inhibits caffeine metabolism by the competitive inhibition mechanism since alarge alpha value means the binding of caffeine to CYP1A2 isozyme is decreased by the binding of theophylline to the same enzymatic sites (Copeland, 2005; Strelow, 2012).

Table 1 summarizes the enzyme kinetic parameters i.e., K_M, V_{max}, IC_{50} and K_i values obtained in the present study. It is important to determine the enzyme kinetic parameters especially the K_i value for the caffeine/theophylline interaction study because the K_i can be used to predict the occurrence of adverse health effects if plasma theophylline concentration is known. In other words, theophylline is likely to cause caffeine metabolism inhibition if the plasma concentration of theophylline is much higher than the Ki value (Kakkar *et al*., 2000).

Table 1. A summary of kinetic parameters calculated from the rate of paraxanthine formation.

Parameters	Values
K_M	0.66 ± 0.06 mM Caffeine
V_{max}	106.3 ± 3.4 ng paraxanthine/hour/mg
IC_{50}	75.8 ± 5.2 μM Theophylline
K_i	0.41 ± 0.03 μM Theophylline

CONCLUSION

This is the first report on *in vitro* inhibition of caffeine metabolism by theophylline. Our results demonstrate that theophylline acts as a competitive inhibitor of caffeine metabolism. Theophylline probably also is able to inhibit the metabolism of other caffeine metabolites such as theobromine (Fig. 1). It is important to study the inhibitory effects of various xenobiotics on CYP1A2 activity since this isozyme is involved in the metabolism of drugs and carcinogens (Landis *et al*., 2018).

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