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Laboratory Exercises

HPLC Determination of Caffeine and Paraxanthine in Urine

AN ASSAY FOR CYTOCHROME P450 1A2 ACTIVITY[S]

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Cytochrome P450 enzymes are a family of heme-containing proteins located throughout the body with roles in metabolism of endogenous and exogenous compounds. Among exogenous compounds, clinically relevant pharmaceutical agents are nearly all metabolized by P450 enzymes. However, the activity of the different cytochrome P450 enzymes varies among individuals and, therefore, so does drug efficacy as well as susceptibility to side effects and toxicity. Thus, assessing P450 activity is of great interest in drug development and clinical pharmacology. This study investigates the phenotyping of a single P450 activity by analyzing urine samples using isocratic reverse-phase HPLC. Specifically, the activity of human P450 1A2, which converts caffeine into paraxanthine, can be investigated by measuring the change in caffeine and paraxanthine concentrations in urine over time following a single dose of caffeine. There is an observable relationship between caffeine intake and paraxanthine formation that varies among individuals. This laboratory exercise provides a means for simple assessment of P450 1A2 metabolic activity using an HPLC method without additional extraction or purification steps and introduces students to the complexities of individualized medicine as well as the basic biochemical techniques of sample preparation and quantitative HPLC. Furthermore, students may design and test their own hypothesis using these methods.

Keywords: Cytochrome P450 enzyme, drug metabolism, caffeine, P450 1A2, HPLC, pharmacokinetics.

Cytochrome P450 enzymes are heme-containing monooxygenases responsible for the metabolism of many endogenously synthesized compounds such as cholesterol, steroid hormones, and fatty acids. Xenobiotics such as pharmaceuticals, food additives, and carcinogens are also known substrates for the P450 family of enzymes [1, 2]. Named for their characteristic absorbance of light at 450 nm when reduced in the presence of carbon monoxide, P450s consist of a family of 57 members in humans and are present in organisms from bacteria to man [2]. The basic reaction catalyzed by P450s is a monooxygenation or mixed-function oxidase reaction

$$NAD(P)H + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH$$

where electrons are provided from NAD(P)H to the heme iron of P450 by P450-NADPH reductase. In the basic reaction, one atom of oxygen is added to the substrate and the other reduced to water. However, because of rearrangement reactions, hydroxylations are not the only observed reactions. Products also form from dealkylation, epoxidation, heteroatom oxygenation and dealkylation, group migration, oxidation of olefins and acetylenes, and heme inactivation reactions and many other atypical reactions. An introduction to P450 enzymes in the context of modern metabolism was recently presented in this journal [2]. The focus of this paper is to introduce instructors and students to an experimental investigation of P450 metabolism in humans, specifically metabolism of caffeine by P450 1A2.

P450 1A2 is present in the liver primarily, but is inducible in the intestine, skin, lymphocytes, placenta, lung, gastrointestinal tract, pancreas, and brain [3, 4]. Besides caffeine, P450 1A2 is involved in the metabolism of several pharmaceutical drugs as well as environmental carcinogens. Pharmaceutical substrates for P450 1A2 include acetaminophen, estradiol, theophylline, warfarin, imipramine, olanzapine, and others. P450 1A2 is also responsible for the electrophilic activation of aromatic and heterocyclic amines and polycyclic aromatic hydrocarbons [5, 6]. Heterocyclic amines, produced during the cooking of red meat for instance, are metabolized by P450 1A2 to electrophilic metabolites that have been shown to cause DNA mutations and cancer [7, 8]. Polycyclic aromatic hydrocarbons, also present in high concentrations in charbroiled meats as well as tobacco smoke, serve both as substrates for and inducers of P450 1A2 activity [9]. Thus, individuals with diets high in char-grilled meat and/or are smokers have been shown to have increased P450 1A2 activity [9–15].

[S] The on-line version of this article (available at http://www.bambed.org) contains additional details concerning the supplementary materials.

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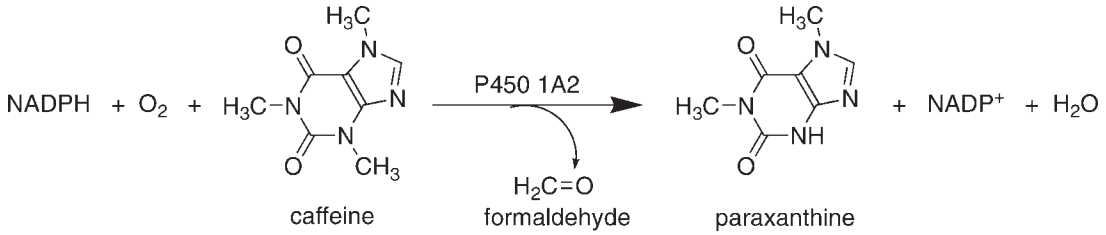

FIG. 1. **3-Demethylation of caffeine (1,3,7-trimethylxanthine) to form paraxanthine (1,7-dimethylxanthine) by P450 1A2.**

Multiple environmental factors have been identified as either inducers or inhibitors of P450 1A2. These include dietary and nutritional factors, hormonal changes in the body, ingestion of medicinal agents, and exposure to environmental factors [7]. Diet is an environmental factor that greatly influences the activity of P450 1A2. In addition to char-grilled meats mentioned earlier, cruciferous vegetables (including broccoli and cauliflower) have an inducing affect on the metabolic activity of P450 1A2 [16, 17]. Apiaceous vegetables (dill weed, celery, parsley, parsnip, carrot) have an inhibitory affect while allium vegetables (garlic, onions, scallions, leeks, and chives) have no effect on P450 1A2 activity when compared with a control diet lacking vegetables [18]. Oral contraceptives and estrogen hormone replacement therapy have also been identified as inhibitors of the metabolic activity of P450 1A2 [11, 15, 19]. Individuals who are exposed to enzyme inducers or inhibitors in the workplace, such as anesthesiologists exposed to anesthetic chemicals in the operating room, gasoline station attendants exposed to high levels of petroleum, and greenhouse workers exposed to pesticides, may also exhibit altered P450 1A2 metabolic activity [20–23]. Beyond these environmental factors, age and gender have also been shown to affect P450 1A2 metabolic activity with children having higher activity than adults, and men having higher activity than women, though this latter observation is disputed [11, 14, 15, 18, 19].

In addition to environmental factors, there are some connections to genetic influence on P450 1A2 metabolic activity. Over 85 single nucleotide polymorphisms (SNPs) in the P450 1A2 gene locus have been identified [4]. Efforts to associate these genotypes with P4501A2 metabolic phenotypes have been ongoing. However, prediction of metabolic phenotype based on genotype with P450 1A2 has proven to be challenging and is an area of research that generates continued controversy. Multiple studies have shown that genotype for P450 1A2 does not appear to result in a specific interindividual variation or phenotype of P450 1A2 metabolic activity [4, 12, 24]. In the end, interindividual differences in phenotypic P450 1A2 activity still remain of great interest because of the connection with drug metabolism and carcinogen metabolism. Indeed, understanding of interindividual differences in pharmacokinetics (the study of the bioavailability of a substance, mainly therapeutic drugs, resulting from its clearance by metabolism) has been a part a recent movement in the direction of personalized drug treatment [25]. The goal of this movement is to provide individuals with medications that relate their phenotype (or genotype when correlated) to the pharmacokinetics of individual drugs [26].

The relationship between the laboratory exercise presented here and these increasingly important topics in medicine and pharmacology should generate significant student interest in completing a laboratory exercise like the one described here. These experiments will also allow for a high level of student independent exploration and introduce mid-to-upper level undergraduate students to the complexity of individualized medicine and biochemical basis for study of pharmacokinetics. While the execution of the experiments described here are straightforward, it is the data analysis and interpretation that will present students the opportunity to explore basic scientific method, drug metabolism, and ethical issues in greater detail. By adding an additional layer of analysis, students with interest in biochemistry and analytical chemistry can relate caffeine metabolism to drug metabolism and pharmacokinetics in the experiment described. This experiment also fits well with discussions of modern metabolism and emphasis on techniques highly recommended by professional societies for biochemistry and molecular biology curricula, namely high performance liquid chromatography (HPLC)[1] [27]. Discussion of HPLC as a powerful, quantitative analytical separation tool in biochemistry would be relevant.

The experimental objective of this laboratory exercise is to determine an individual's P450 1A2 phenotypic activity by use of HPLC analysis of caffeine and a primary metabolite present in urine. Caffeine is a naturally occurring, nonpolar plant alkaloid. It has been identified as an appropriate probe for measuring the activity of P450 1A2 because of its rapid and complete absorption throughout the total body water, its low plasma protein binding, its complete biotransformation in the liver, and negligible renal excretion [2, 19, 28, 29]. In fact, many pharmaceuticals have been analyzed for drug specific phenotyping of P450 1A2 alongside caffeine [30]. Caffeine is 3-demethylated by P450 1A2 in the liver to form paraxanthine and formaldehyde (Fig. 1) and the mechanism for this reaction is well understood [1]. In this labortory experience, both caffeine and paraxanthine present in urine are determined and used to estimate the metabolic activity of P450 1A2 in different individuals [14, 31].

The laboratory exercise presented here combines skills from many different disciplines, making it an appropriate lesson for chemistry, biology, or biochemistry students as well as pre-medicine or pharmacy programs. The student learning objectives for this laboratory experience include: introduction to the P450 family of enzymes and metabo-

[1] The abbreviations used are: HPLC, high performance liquid chromatography; P450, cytochrome P450 enzyme.

lism of xenobiotics; HPLC as a biochemical separation tool related to other forms of chromatography common in a biochemistry laboratory; environmental and genetic factors leading to the induction/inhibition of an important enzyme (P450 1A2) involved in metabolism (there is a vast literature in this area available for student exploration); and the growing use of pharmacokinetics in the clinical diagnostic and medical fields. This experiment has been performed successfully with several student groups at Kalamazoo College with positive response. A collection of student reactions are provided in the *Supplemental Materials* available online for this paper.

EXPERIMENTAL METHODS

Detailed guide to laboratory set-up is available as *Supplemental Materials*.

Supplies—Ethanol, acetonitrile (HPLC grade), ammonium acetate, glacial acetic acid, caffeine, and paraxanthine were obtained from Sigma-Aldrich (St. Louis, MO). Water, 18 megaohm, filtered using a Millipore purification system was used. All solvents and samples were filtered using a 0.45 μm filter prior to use with HPLC.

Required Equipment—HPLC system (isocratic capabilities), micropipette set, 0.45 μm filters (1 mL filtering size or smaller), disposable syringes for filtering samples, refrigerated centrifuge, urine collection cups, disposable centrifuge tubes, and pH meter or pH paper.

Standard Solutions—Paraxanthine and caffeine stock solutions were prepared by dissolving 2.5 mg and 5.0 mg respectively of each in 5.00 mL of water-ethanol (50:50, v/v). Dilution of each stock solution in duplicate with 0.01 *M* ammonium acetate solution gave standard solutions within the range of 0.5–10 μg/mL. Standard solutions were then filtered using a 0.45 μm filter and stored at 4°C.

Sample Collection—Participants for this study were recruited from among students and faculty at the college following protocols approved by the Institutional Review Board of Kalamazoo College (sample Consent Form is included in the *Supplemental Materials*). Please note that instructors should be mindful to obtain Institutional Review Board approval at their institution for research with human subjects, particularly if volunteers outside of the class are recruited to participate. Identities of all volunteers should be kept confidential and a blind participant numbering system should be used.

Participants were asked to abstain from the consumption of caffeinated foods and beverages for a time period of 16 hr. In addition to caffeinated products participants were asked to abstain from the following products that might alter P450 1A2 activity: cruciferous vegetables (broccoli, cauliflower), charbroiled meats, smoked foods, watercress, and red wine. Following the consumption of two 12 oz. Mountain Dew® beverages (total of 110 mg caffeine), participants collected their urine at the time of consumption (t_0), and at 3 and 6 hr following caffeine intake. Samples were stored at 4 °C and prepared as described later within 24 hr of receiving them. Though not part of the study design, all participants were non-smokers.

Sample Preparation—Each urine sample was adjusted to a pH of 3.1 with stirring by addition of glacial acetic acid (pH paper can be used for acidification verification). For each sample, 5 mL was removed and centrifuged for 10 min at 3,800 rpm and 4 °C. Approximately 1 mL of the supernatant was then filtered using a 0.45 μm filter and stored at 4 °C for analysis by HPLC. Students who may be uncomfortable working with human urine may choose to use synthetic urine spiked with paraxanthine and caffeine (http://www.dtitesting.com/).

Compound Separation—Reverse phase HPLC separation was performed using a Waters/Alliance 2690 Separations Module in conjunction with a Water 2487 dual wavelength absorbance detector (Waters Corp., Milford, MA). A Waters Symmetry C_{18}, 5 μm (3.9 × 150 mm^2) column was used for separation. The mobile phase consisted of a 90:10 ammonium acetate (0.01 *M*, 2.5% glacial acetic acid)/acetonitrile solution and had a flow rate of 1.0 mL/min. The sample chamber of the HPLC autosampler was set to 4 °C for the duration of the run time. Sample and standard injections were 20 μL. Chromatograms were visualized using Waters Millennium 32 Software on an E-3400 Gateway Computer.

Hazards—Because of the use of human urine samples, instructors must discuss the possible hazards of working with a biological fluid. Use of protective goggles, gloves, and a lab coat, and use of 5% bleach solution to clean all affected glassware and bench areas is required. Though the present study utilized urine samples from study volunteers, in a class setting, students could analyze their own samples.

RESULTS AND DISCUSSION

In this laboratory exercise the phenotypic activity of P450 1A2 within different individuals was determined using a reliable and noninvasive procedure. Caffeine and paraxanthine standard solutions were analyzed as outlined in the experimental methods. A plot of the integrated peak areas versus the known concentrations of paraxanthine and caffeine yielded calibration curves that were used in the calculation of urinary concentrations of caffeine and paraxanthine (Fig. 2*A* and *B*). The calibration curves were each linear with coefficients of correlation $>$ 0.99 over a concentration range of 0.5–10 μg/mL. The average retention times for paraxanthine and caffeine were determined to be 2.5 min and 4.1 min, respectively.

Urine samples obtained from ten study participants were analyzed using HPLC according to the procedures described previously. Whole and expanded chromatograms of the raw urine samples identified multiple peaks in the expected elution regions of paraxanthine and caffeine. Representative HPLC elution profiles are shown in Fig. 3. To confirm peak identity, each urine sample was then spiked with caffeine and paraxanthine (representative chromatograph in Fig. 3C). Paraxanthine was detected in all collected urine samples and ranged in concentration from 1.93 to 51.98 μg/mL (Table I). Caffeine was detected in 23 of the 30 urine samples collected and ranged in concentration from 0.70 to 77.17 μg/mL (Table I). Detected caffeine concentrations were normally smaller than those of paraxanthine and the lowest limit of detection of caffeine or paraxanthine in urine samples was 0.5 μg/mL.

The metabolic ratio comparing the concentration of paraxanthine to caffeine within each sample was determined using the relationship MR = [paraxanthine]/[caffeine] as described by Kadulbar *et al.* [31] (Fig. 4). The range for MR values found was 0.34 and 26.34 (Table I)

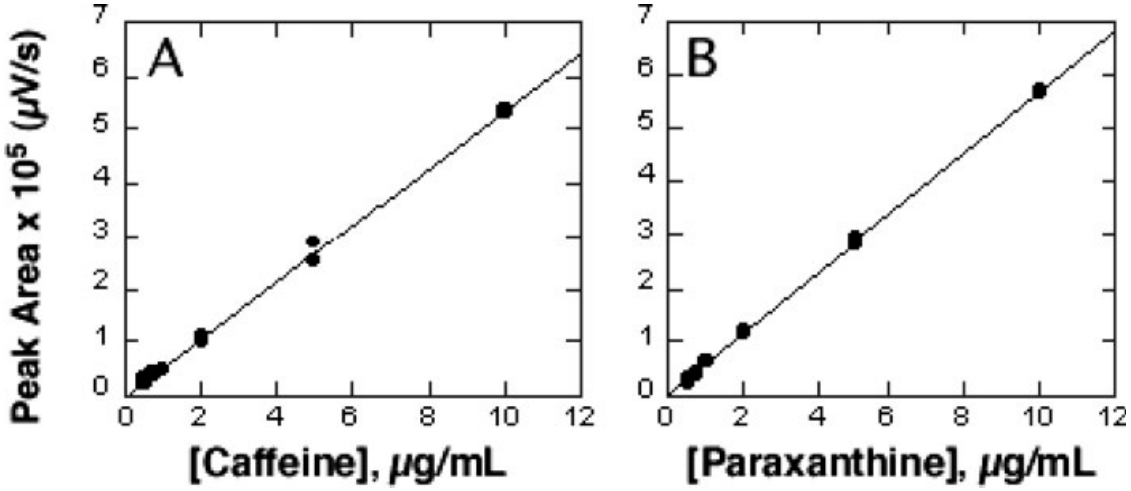

FIG. 2. **Standard curves.** (*A*) A linear fit of the caffeine standards yielded a calibration curve with equation: Peak Area = 53869 μV*mL/μg*s (concentration) + 440.96 μV/s. (*B*) A linear fit of the data yielded a calibration curve with equation: Peak Area = 56582 μV*mL/μg*s (concentration) + 5903.1 μV/s.

and these values are within the ranges described in the literature by many groups and with much larger sample sizes (MR values between 0.27 to 21.24 are common [30, 32, 33]).

The largest percentage of our study participants (4 of 10) were determined to have the greatest value for MR at the 3-hr time point, consistent with rapid caffeine metabolism by P450 1A2. For samples with caffeine concentrations below detection levels, no MR values were calculated. However, it is expected that the MR values for these individuals would be high. Because of the small sample set of this study no definitive conclusions could be made as to the affect of gender, race, age, diet, or average number of caffeinated beverages per week on the metabolic activity of P450 1A2. Furthermore, all study participants were non-smokers precluding any analysis of differences between smokers and non-smokers.

Multiple study participants had detectable caffeine concentrations at the 0 hr time mark and one study participant had the greatest MR at this point which suggests noncompliance with the experimental procedure. This may have been the result of participants not following the preconsumption protocol such as consuming caffeinated beverages, smoking, or eating foods that affect the metabolic activity of P450 1A2. Another possible reason for this phenomenon could be that these individuals are slow P450 1A2 metabolizers and that 16 hr was not enough time for the complete metabolism of caffeine, though we would have expected a peak of caffeine soon after the consumption of 110 mg of caffeine and we did not observe one. Another limitation in this experimental protocol is urine volume and levels of dilution determined by how much water study participants consumed as well as height and weight. These factors will affect the concentrations of caffeine and paraxanthine present in the sample thus leading to differences in the measured MR and there are examples in the literature where a much more rigid collection of urine samples that accounts for volume as well as height and weight of study participant [32]. However, we found that our MR values were very similar to literature values and the methods described

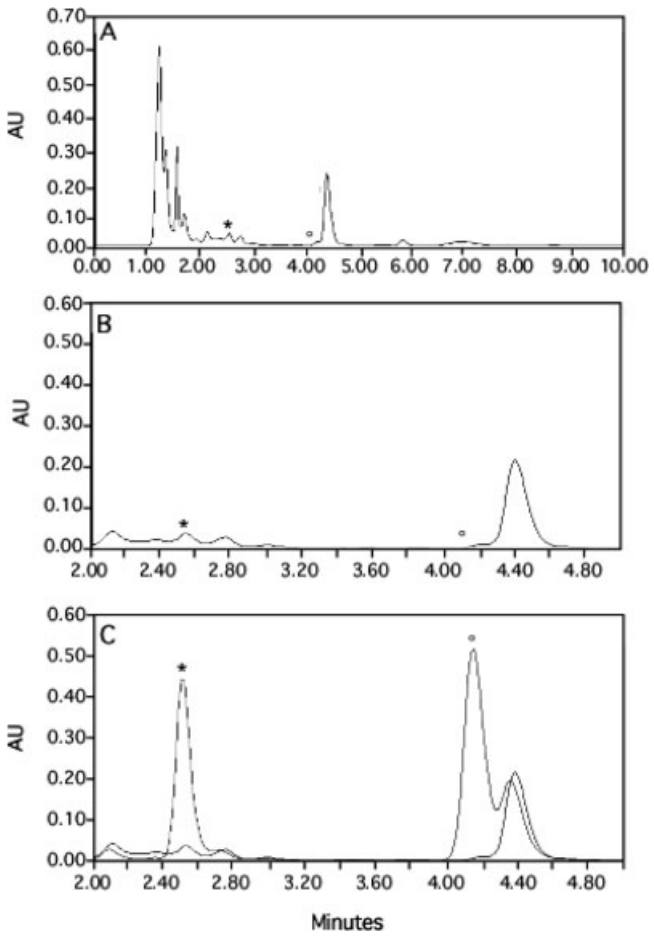

FIG. 3. **Representative HPLC chromatograms of urine samples from subject 15896.** The expected position of paraxanthine is indicated with (*) and caffeine with (°). (*A*) A whole chromatogram of a urine sample obtained at the 3 hr time point. (*B*) An expanded chromatogram (2–5 min) of the region containing paraxanthine and caffeine. (*C*) Overlay of the chromatogram shown in part B with an expanded chromatogram of the same sample spiked with caffeine and paraxanthine.

TABLE I
Tabulated metabolic ratios for P450 1A2 activity

Subject ID	Relative time	Concentration in the sample (μg/mL)		Metabolic ratio
		Caffeine	Paraxanthine	
15247 (0)	0.08	77.12	26.42	0.34
15247 (3)	3.08	2.86	18.32	6.39
15247 (6)	6.02	3.65	30.88	8.47
19824 (0)	0.12	4.98	14.96	3.01
19824 (3)	2.98	2.38	7.62	3.20
19824 (6)	6.00	5.36	12.78	2.38
12478 (0)	0.50	3.23	51.98	16.10
12478 (3)	2.75	2.73	6.24	2.29
12478 (6)	6.75	7.06	20.54	2.91
18652 (0)	0.50	nd*	4.43	–
18652 (3)	3.00	2.40	1.93	0.81
18652 (6)	5.50	3.39	2.71	0.80
15896 (0)	0.08	nd	9.31	–
15896 (3)	2.83	1.46	5.42	3.73
15896 (6)	5.38	2.55	12.09	4.75
15764 (0)	0.16	nd	26.42	–
15764 (3)	3.12	0.70	18.32	26.34
15764 (6)	na	nd	30.88	–
14789 (0)	0.08	4.56	17.89	3.92
14789 (3)	3.20	nd	23.22	–
14789 (6)	5.98	8.91	21.81	2.45
19548 (0)	0.08	5.15	26.73	5.19
19548 (3)	3.08	nd	29.32	–
19548 (6)	6.08	10.99	36.32	3.30
18962 (0)	0.02	nd	4.97	–
18962 (3)	3.80	2.17	4.21	1.94
18962 (6)	5.92	5.88	11.39	1.94
12684 (0)	0.25	2.98	5.25	1.76
12684 (3)	3.50	2.34	7.30	3.12
12684 (6)	6.00	3.00	15.22	5.07

All study participants were non-smokers. nd: not detected.

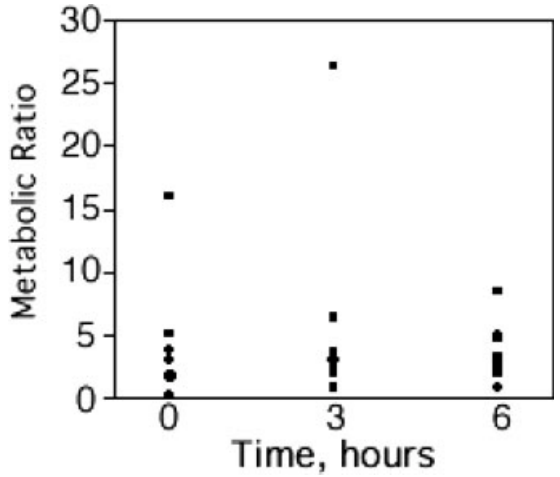

FIG. 4. **Distribution of P450 1A2 caffeine metabolic ratios.** The metabolic ratio of each urine sample at each time point was determined using the relationship MR = [paraxanthine]/[caffeine].

here are nearly identical to those used with research studies of P450 1A2 phenotype and drug metabolism or phenotyping/genotyping associations in the primary literature. Finally, there are two other minor metabolites of caffeine, theobromine and theophylline, and both of these compounds as well as paraxanthine are converted into other metabolites in secondary reactions. Though these alternative fates of caffeine are on average minor, the production of metabolites other than paraxanthine can cause MR values to vary [32]. Likewise, while P450 1A2 has been shown extensively *in vitro* with human liver microsomes and in human populations to be the primary enzyme involved in caffeine metabolism [13, 33, 34], there may be minor additional interindividual differences in caffeine metabolism that may not be accounted for in this analysis. Overall, the discussion of limitations of any experimental procedure is of use in the undergraduate education of a student in all fields of science.

There are multiple ways to adapt the protocol presented here. Students may choose to further explore the effects of environmental factors on the metabolic activity of P450 1A2. This could be accomplished by adjusting their diets or smoking habits over two or more conditions. For example, students could ingest broccoli prior to the ingestion of caffeine and investigate the differences in P450 metabolic activity. Alternatively, a lunch including a hamburger or other char-grilled alternatives could be served 1 hr before the consumption of caffeine. Students who are smokers could refrain from smoking for a time period of 24 hr prior to the ingestion of caffeine. The sample analyzed could also be changed to saliva with similar workup to the urine sample [35]. Students could also generate pharmacokinetic plots that relate time to decreases in caffeine concentration by taking more urine samples over a longer time period (24 hr, for instance). To connect biochemistry and molecular biology disciplines and analytical techniques, it is possible that students could correlate phenotypic results with the methods presented here with genotype assays following restriction fragment length polymorphism (RFLP) protocols described elsewhere in the literature for P450 1A2.

CONCLUDING REMARKS

The trend towards individualized medicine requires knowledge of an individual's metabolic profile. For some metabolic enzymes, this can be predicted by genotype; for others, such as P450 1A2, the genotype does not allow for prediction of phenotype [4]. Given the role of P450 1A2 in metabolism of several important pharmaceutical drugs and in activation of chemical carcinogens, there is great interest in methods to determine an individual's P450 1A2 phenotype. Phenotyping P450 1A2 activity by students as part of a biochemistry or molecular biology curriculum is an opportunity for students to become familiar with complex issues in modern metabolism and medicine as well as understand and apply standard biochemical laboratory techniques. Indeed, students that have completed this lab have commented specifically on the benefits of designing their own experiment, preparing a lab report in the format of journal article (information relating to report preparation are available as *Supplemental Materials*), working with HPLC, interesting their nonscience major friends in science and metabolism (many of the study volunteers were nonscience majors that were friends with the students conducting the analysis), and exploring medically relevant topics beyond traditional metabolism. It should be emphasized to students prior to the start of these experi-

ments that the results of MR for P450 1A2 do not reveal superiority or inferiority in any way and that all individuals display enormously different phenotypes for metabolic pathways.

As presented here, the experimental analysis can be completed in one 3-hr laboratory class if each individual analyzes their own samples that they have collected outside of class time. If students collect and analyze a larger group of samples, two 3-hr laboratory class periods should be reserved for completion of the experiment. Other sample preparation methods have required an extraction step before the analysis of urine metabolites could be performed [19, 15, 36]. However, we have found our analysis to be satisfactory without extraction as described here, thus allowing for a shorter laboratory time commitment and purchase of fewer reagents. Extraction with a chloroform/isopropanol mixture could be included if greater focus on analytical techniques is desired. Previous studies have also assessed the MR of P450 1A2 by analyzing paraxanthine as well as its secondary metabolites, leading to a larger data analysis time commitment. The procedure described here obtained results similar to those that have been presented before and therefore is a reliable analysis of the caffeine metabolic ratio even though it does not account for the other minor caffeine metabolites or metabolites of paraxanthine [11, 19, 30, 32].

In summary, the data presented here demonstrates that HPLC analysis of urine samples utilizing a caffeine probe is a reliable method for mapping the metabolic activity of P450 1A2. The laboratory exercise and data analysis presented here were successfully completed by multiple student groups at our institution as part of an advanced topics course. Because of the vast literature in this area, and interesting controversy regarding phenotype versus genotype for P450 1A2 activity, this exercise can provide a springboard to critical discussions of the primary literature and of progress in individualized medicine.

Acknowledgments— In memory of Sarah Klenow. We thank the various volunteers who participated in this study as well as the students who have helped work out the conditions and analysis presented in this laboratory, particularly Sarah Klenow, Emery Engers, Michael Mequio, Rachel Sherman, Jamie Chun, and Emilie Manz. We gratefully acknowledge the valuable technical support for maintenance of our HPLC systems by Waters Corporation.

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