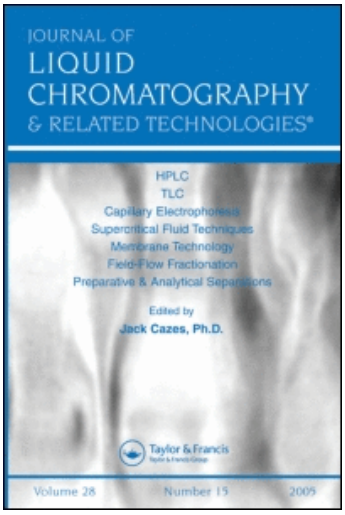

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Measurement of Caffeine Metabolites in Urine Using Diode-Array Detection HPLC

Joseph N. Miceli[a]; Winifred Chapman[a]
[a] Departments of Pharmacology and Medicine, Morehouse School of Medicine, Atlanta, Georgia

To cite this Article Miceli, Joseph N. and Chapman, Winifred(1990) 'Measurement of Caffeine Metabolites in Urine Using Diode-Array Detection HPLC', Journal of Liquid Chromatography & Related Technologies, 13: 11, 2239 — 2251
To link to this Article: DOI: 10.1080/01483919008049027
URL: http://dx.doi.org/10.1080/01483919008049027

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(11), 2239-2251 (1990)

MEASUREMENT OF CAFFEINE METABOLITES IN URINE USING DIODE-ARRAY DETECTION HPLC

JOSEPH N. MICELI AND WINIFRED CHAPMAN
Departments of Pharmacology and Medicine
Morehouse School of Medicine
Atlanta, Georgia 30310-1495

ABSTRACT

Diode-array detection high performance liquid chromatography (HPLC) offers significant advantages when compared to conventional UV HPLC detection. These advantages include important spectral information such as the τ max and the UV spectra of each eluting peak. The spectral scans are obtained at the peak start, peak apex and peak end. This provides a comparison of these spectra so that peak homogeneity can be calculated. Standard spectra can be stored and compared to eluting peaks for "purity". Therefore, peak identification is based not only on retention time but the aforementioned spectral information.

Recently, pathways involved in the metabolism of caffeine have been determined. One of the pathways involves the N-acetyltransferase system. Thus, a safe non-invasive and convenient procedure is now available to determine the N-acetylation phenotype. This is important because a number of toxicities have been associated with acetylator phenotype.

The present paper indicates the utility of the diode-array detector for performing these studies. Use of this detector provides greater accuracy and reliability in correctly identifying the key metabolites required for classification as compared to conventional detection HPLC.

INTRODUCTION

N-acetyltransferase catalyzes the acetylation of a number of clinically important drugs including the sulfonamides and isoniazid. Acetylation capacity is an inherited trait that exhibits genetic polymorphism in humans. Humans can be classified as homozygous rapid, heterozygous rapid (intermediate) and homozygous slow acetylators based on their ability to acetylate a test drug (1,2).

Acetylation status has been linked to a number of toxicities and disease entities. Examples include: the association between slow acetylation and (a) isoniazid-induced neuritis (3,4); (b) hydralazine-induced lupus erythematosus (5,6); (c) procainamide-induced lupus (7,8); and (d) isoniazid-phenytoin-induced toxicity (9). On the other hand, it appears that rapid acetylators have an increased risk of bladder cancer (10), colorectal carcinomas (11,12) and breast cancer (13,14). Although there are conflicting reports, it appears that there is a greater preponderance of rapid acetylators in insulin dependent diabetics and no relationship in non-insulin dependent diabetics as compared to control populations (15-21).

In order to accurately phenotype an individual a test drug is administered, usually a sulfonamide or isoniazid, and blood samples obtained at appropriate times. These drugs can produce severe side-effects and their use for studies in otherwise healthy children is unethical. Thus, our knowledge of acetylation capacity and in disease status such as lupus, is extremely limited by the use of the test drugs (22,23).

During the past few years, Grant and co-workers (24,25) have described the use of caffeine for determination of the acetylator phenotype. Caffeine (1,3,7 trimethylxanthine) undergoes 3-demethylation to form 1,7 dimethylxanthine (17X) which undergoes subsequent 7-demethylation to form 1 methylxanthine (1X). The pathway for evaluation of the acetylator phenotype involves the formation of 5-acetylamino-6-formylamino-3-methyluracil (AFMU) from a putative cyclic intermediate that apparently forms during the conversion of 17X to 1X. The ratio of AFMU:1X can then be used as a means of acetylation classification; the higher the ratio, the faster the acetylation. Briefly, their procedure can be summarized as follows: Each subject is given a cup of coffee or other caffeine-containing

beverage and a urine sample is collected 2-4 hours later. The sample is then analyzed for the caffeine metabolites (AFMU, 1X) using HPLC. The purpose of this paper is to describe the utility of the diode array detector in this procedure which results in greater accuracy, reliability and confidence in measuring these metabolites and reduces the possibility of acetylation misclassification.

MATERIALS AND METHODS

A Perkin-Elmer HPLC (Perkin-Elmer Corp., Norwalk, Ct.), consisting of a model 410 pumping system, LCI-100 integrating recorder and model 235 diode-array detector, was used for the analysis. The column was a Hibar Lichrosorb RP-C18 column (E.M. Merck, New Jersey) and it was maintained at room temperature during the analysis. All common laboratory chemicals were reagent grade and solvents were HPLC grade (either Fischer, Atlanta, GA or Burdick and Jackson, Muskeegon, MI). Caffeine metabolites, including 1X and 17X, the corresponding uric acid metabolites, (1U, 17U) and the internal standard, parahydroxybenzoic acid (PHBA), were obtained from Sigma (Sigma Chem. Co., St. Louis, Mo.). AFMU was obtained from Dr. Tang (Toronto, Canada).

Stock standards of each xanthine and urate were individually prepared in distilled and deionized water. A few drops of 0.1N NaOH was added to each metabolite to facilitate solution. AFMU was not placed in alkaline media at any time. Dilutions were prepared so that working standards of 100 ug/ml were used to chromatographically identify the retention time of each metabolite. A composite standard was also prepared to determine chromatographic resolution of peaks.

Caffeine-free urine was obtained from a number of human volunteers who abstained from caffeine-containing food or beverage for five days. Endogenous peaks were usually present upon

chromatographic analysis (see below). Blank urine samples were fortified with the caffeine metabolites as described above for the water samples. Final concentrations studied for 1X and AFMU were 25, 50 and 100 ug/ml of each.

The samples were treated as follows: 0.2 ml of water or urine was placed in a 15 ml glass screw-capped conical centrifuge tube that contained 0.1 ml concentrated phosphoric acid. Fifty (50) ul of the internal standard solution (25 ug/ml) was added. The sample was then extracted with 6 ml chloroform: isopropanol (95:5, v:v), vortexed for about 30 seconds and extracted by a rotary test tube extractor. The samples were then centrifuged (10 min, 2500 g) and the organic layer transferred to a clean, dry test tube. The extract was taken to dryness at 40°C under nitrogen. The residue was reconstituted with 100 ul of mobile phase (0.05% acetic acid in water: acetonitrile, 95:5, v:v) and 10 ul was injected onto the column. The flow rate was 1.0 ml/min and the effluent was monitored at 280 mm.

Two individuals volunteered to participate in a study to assess the validity of the method. A "test" urine sample was obtained any time after two hours of consumption of an appropriate volume of either coffee, tea or cola beverage (test dose of caffeine equal to about 1.5 mg/kg). Collected samples were stored frozen (-20°C) under acidic conditions until analysis. These samples were treated as decribed above.

RESULTS

Figure 1 shows a typical chromatogram obtained from a "spiked" water sample. The retention times for the 1X, AFMU and PHBA peaks were 3.7, 7.3 and 18.0 minutes, respectively. Spectral characterization of the AFMU and 1X peaks are shown in figures 2 and 3. Figure 4 shows a chromatogram of a caffeine-free urine sample

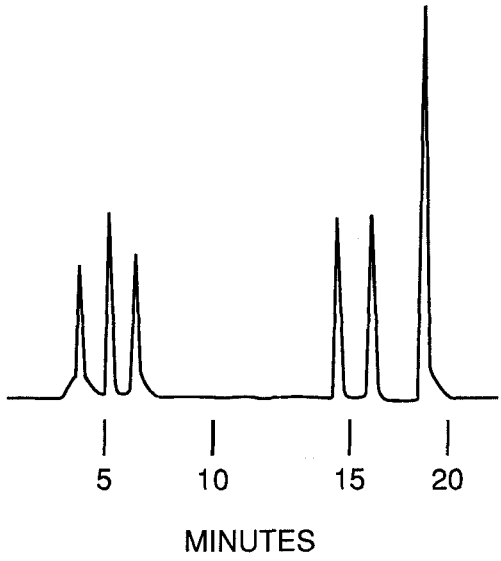

Figure 1. A chromatogram obtained after analysis of a water sample fortified with the most abundent caffeine metabolites produced by humans. The metabolites and the internal standard (BHET), listed in increasing order of elution time, are: AFMU (5-acetylamino-6-formylamino-3-methyluracil, 3.7 min); 1U (1-methyluric acid, 5.8 min); 1X (1-methylxanthine, 7.3 min); 17X (1, 7 dimethylxanthine, 14.7 min); 17U (1,7 dimethyluric acid, 15.7 min); and BHET, (parahydroxybenzoic acid, 18.0 min). The τ max for each respective peak was 284, 285, 268, 270, 290 and 255 nm). The concentration for each metabolite was 100 ug/ml.

fortified with the caffeine metabolites and PHBA. There were no interfering peaks found in caffeine-free urine. Figure 5 shows a chromatogram obtained from a test consumption study. All samples were processed as described above. In order to assess day to day and within run precision, three separate samples of 1X and AFMU, each containing 100 ug/ml, 50 ug/ml, and 25 ug/ml were prepared in

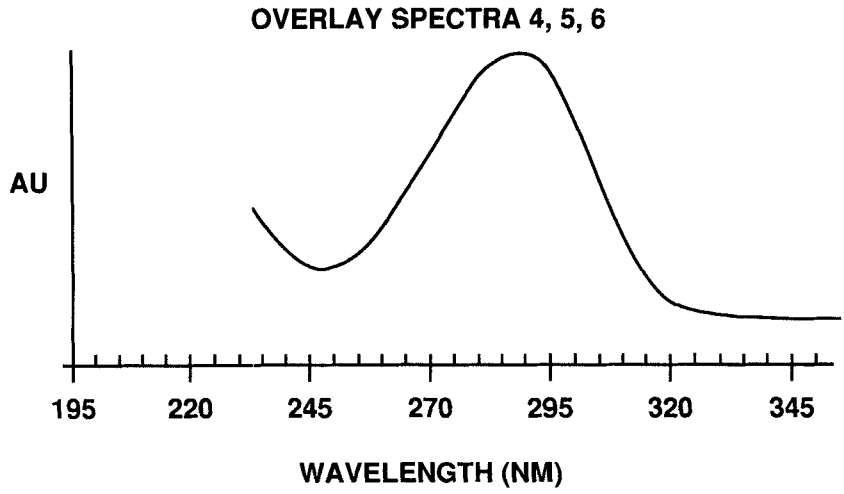

Figure 2. Spectral scan of the AFMU standard. The τ max was 284 mm. The plot shows the "overlay" of spectra 4, 5 and 6. These spectra were collected at the peak start, peak apex and peak end. The single line indicatess no discrepencies in these three spectra; spectral "purity" of the peak was 1.0.

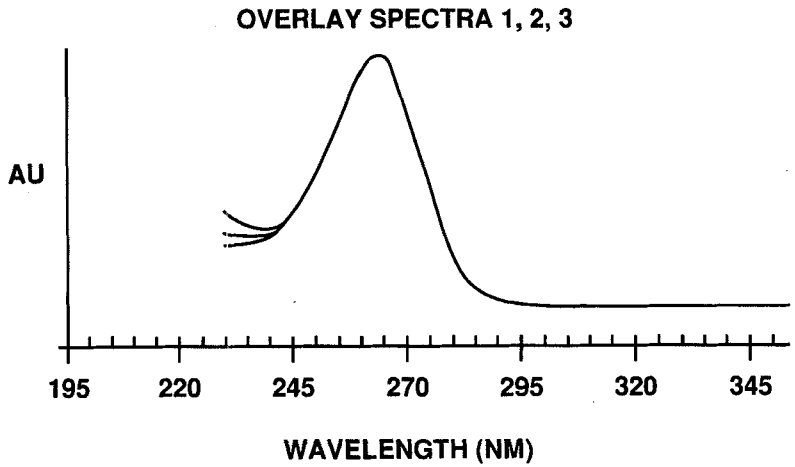

Figure 3. Spectral scan of the 1X standard. The τ max was 267 mm. The spectral scans indicate purity as described for figure 2.

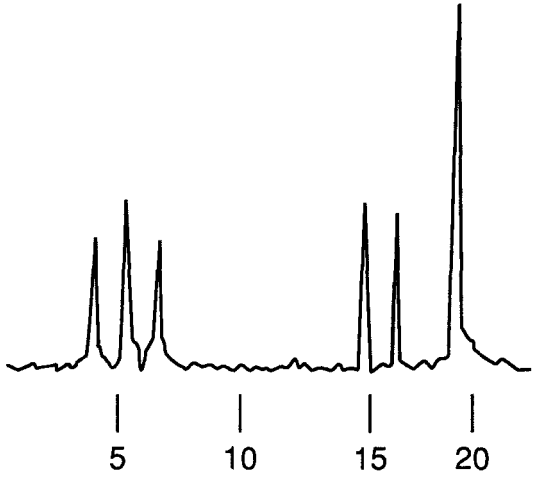

Figure 4. Chromatogram of caffeine-free urine fortified with 100 ug/ml of each of the caffeine metabolites and containing 25 ug/ml PHBA, the internal standard. The retention times for each metabolite and the τ max were the same as described in Figure 1. The purity index for each metabolite was less than 2. The purity index for the internal standard was 1.

caffeine-free urine. The results of the analysis are presented in Table 1. The values indicated for each day are the mean values of the triplicate assay for that day. Each of the concentrations had a calculated CV less than 10 % for day to day precision.

Each of the concentrations had a calculated CV less than 10%

Three samples of each concentration were analyzed on each day. The results of 15 analyses at each concentration were used to calculate the day to day precision shown in the table. As to be expected, greater variation occurred at lower concentrations.

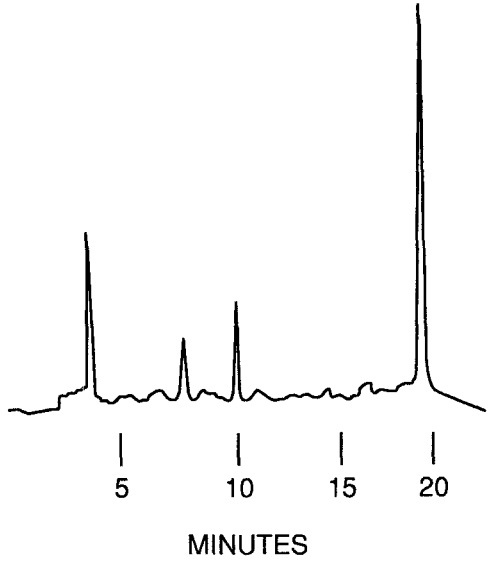

Figure 5. Chromatogram obtained after human consumption of a test dose of caffeine. The AFMU and 1X peaks each had a purity index of 1.5. The purity index for the internal standard was 1. Under our classification system, this individual was classed as a rapid acetylator, which was consistent with a previously obtained sulfamethazine classification.

DISCUSSION

Analysis of urinary caffeine metabolites, as originally described (24,25), based identification solely on retention time. This could present problems because of the presence of caffeine urates, xanthines and other endogenous compounds that are excreted in urine and subsequently extracted. Many of these compounds chromatograph with small differences in retention times. The use of acetaminophen as the internal standard is cause for concern because it is a compound that is present in many over the counter

medications. Thus, erroneous concentration results may be determined when using acetaminophen because acetylation phenotype is based on the AFMU:1X ratio; inaccurate quantitation of either one will cause misclassification.

Due to these concerns, we elected to use a different column, the diode array detector and a different internal standard, parahydroxybenzoic acid (PHBA). This compound is not available for human consumption. In our hands, the use of the Hibar colu.. provided better resolution of the various compounds than has been previously reported using other reversed phase columns. However, identification of peaks was based not only on comparison of retention times with pure standards; but identification was also based on each metabolite's spectral information.

The Perkin-Elmer model 235 diode array detector has the capability of obtaining a spectral scan of each metabolite (this can be archived for future use in spectral comparisons), printing the τ max and determining the "purity" index of each eluting peak. The purity index is calculated according to an algorythm by comparing the spectral characteristics at the start of the eluting peak, at its apex, and at a point just before the peak stops eluting. These spectra are "over-laid" and a number calculated: (a calculated value of 1 indicates spectral purity). Contamination of the peak by co-eluting substances is indicated by an increasing numerical value of the purity index. Calculated values between 1 and 2 indicate a high degree of spectral purity. A calculated value of 2.5 indicates moderate impurity and values above 5 indicate no purity, based on the above described criteria.

Peak quantitation is based on either the measurement of peak height or area relative to that of the internal standard. If either are in error, inaccuracies will result. The diode array detector

TABLE 1

Within Run and Day to Day Precision

	AFMU (ug/mL)			1X (ug/mL)		
Day	100	50	25	100	50	25
1	103.31	46.33	26.66	101.16	47.79	27.20
2	97.08	60.38	26.39	98.83	56.77	30.52
3	100.28	49.28	24.25	100.51	46.25	24.59
4	100.12	53.46	21.18	100.01	51.92	25.61
5	100.21	48.48	27.19	99.42	48.48	24.43
Mean	100.20	51.59	25.13	99.99	50.24	26.47
SD	1.97	4.97	2.22	0.82	3.75	2.25
CV(%)	1.97	9.63	8.82	0.81	7.47	8.51

provides more information for the positive identification of compounds than conventional UV detection HPLC. Thus, diode array detection is far superior to detection and quantitation based solely on retention time.

In order to accurately identify and quantitate the 1X and AFMU metabolites, we used the diode array detector in the following manner. Pure standards were prepared and individually chromatographed so that retention time and spectras were obtained and stored. Subsequent to this, mixtures of the metabolites were prepared in caffeine-free urine and metabolites identified based on their retention times and τ max. Peaks corresponding to the retention time and τ max for AFMU and 1X were spectrally compared to stored, known standard spectra. This comparison, in conjunction with the calculated purity index value, provided greater accuracy and reliability in the identification and quantitation of these two metabolites. This, in

turn, provided greater accuracy and reliability in classification of subjects. This has been confirmed in preliminary studies in which the caffeine method was used to phenotype two individuals previously phenotyped (1 rapid and 1 slow) using sulfamethazine. Additional studies are in progress to assess the reliability in a larger population.

In conclusion, the use of caffeine as a test substrate for studying drug metabolism is ideally suited for the pediatric population because it is a non-invasive procedure. The use of the diode array detector, coupled with an electronic integration system, provides greater accuracy, reliability and reproducibility for correctly phenotyping individuals for their acetylation capacity as compared to conventional detection HPLC analysis.
chromatogram obtained from a test consumption study. All samples were processed as described above. In order to assess day to day and within run precision, three separate samples of 1X and AFMU, each containing 100 ug/ml, 50 ug/ml, and 25 ug/ml were prepared in caffeine-free urine. The results of the analysis are presented in Table 1. The values indicated for each day are the mean values of the triplicate assay for that day. Each of the concentrations had a calculated CV less than 10 % for day to day precision.

Acknowledgment: The authors would like to thank Ms. Angela Clark for secretarial support and Ms. Diana Vono for preparing the figures.
Supported in part by: NIH RR03034

REFERENCES

1. Weber, W.W. and Hein, D.W.: Clinical pharmacokinetics of isoniazid. Clin. Pharmacokinet. 4:401-422, 1979.

2. Weber, W.W. and Hein, D.W.: N-acetylation pharmacogenetics. Pharmacol. Rev. 37: 25-79, 1985.

3. Hughes, H.B., Schmidt, H. and Biehl, J.P.: The metabolism of isoniazid: its implications in therapeutic use. Trans. Conf. Chemother. Tuberc. 14: 217-222, 1955.

4. Davadatta, S., Gangadharam, P.R.J., Andrews, R.H., Fox, W., Ramakrishnan, C.V., Selkon, J.B. and Velu, S.: Peripheral neuritis due to isoniazid. Bull WHO 23: 587-598, 1960.

5. Perry, H.M., Tan, E.M., Carmody, S. and Sakamoto, A.: Relationship of acetyl transferase activity to aninuclear antibodies and toxic symptoms in hypertensive patients treated with hydralazine. J. Lab. Clin. Med. 76: 114-125, 1970.

6. Perry, H.M.: Late toxicity to hydralazine resembling systemic lupus erythematosus or rheumatoid arthritis. Am. J. Med. 54: 58-72, 1973.

7. Henningsen, N.C., Cederberg, A., Hanson, A. and Johnson, B.W.: Effects of long-term treatment with procainamide. Acta Med. Scand. 198: 475-482, 1975.

8. Woosley, R.L., Drayer, D.E., Reidenberg, M.M., Nies, A.S., Carr, K. and Oates, J.A.: Effect of acetylator phenotype on the rate at which procainamide induces antinuclear antibodies and the lupus syndrome. N. Engl. J. Med. 298: 1157-1159, 1978.

9. Brennan, R.W., Dehejia, B., Kutt, H., Verebely, K., and McDowell, F.: Diphenylhydantoin intoxication attendant to slow inactivation of soniazid. Neurology 20: 687-693, 1970.

10. Hein, D.W.: Acetylator genotype and arylamine-induced carcinogenesis. Biochim. Biophysica Acta, 948: 37-66, 1988.

11. Lang, N.P., Chu, D.Z.J., Hunter, C.F., Kendall, D.C., Flammang, T.J. and Kadlubar, F.F.: Role of aromatic amineacetyltransferase in human colorectal cancer. Arch. Surg. 121: 1259-1261, 1986.

12. Ilett, K.F., David, B.M., Detchon, P. and Castlede, W.M.: Acetylator phenotype in colorectal-carcinoma. Cancer Res. 47: 1466-1969, 1987.

13. Bulovskaya, L.N., Krupkin, R.G., Bochina, T.A. Shipkova, A.A. and Parlova, M.W.: Acetylator phenotype in patients with breast cancer. Onocology 35: 185-188, 1978.

14. Cartwright, R.A.: In, Genetic variability in responses to chemical exposure. G.S. Omenn, H.V. Gelboin, eds., Vol. 16, 359-368, 1984. Banbury Report, Cold Spring Harbor Laboratory.

15. Mattila, M.J. and Tiitinen, H.: The rate of isoniazid inactivation in Finish diabetic and non-diabetic patients. Ann. Med. Exp. Fenn. 45:423-427, 1967.

16. McLaren, E.H., Burden, A.C. and Moorhead, P.J.: Acetylator phenotype in diabetic neuropathy. Brit. Med. J. 2: 291-293, 1977.

17. Burrows, A.W., Hockaday, T.D.R., Mann, J.I. and Taylor, J.G.: Diabetic dimorphism according to acetylator status. Brit. Med. J. 1: 208-210, 1978.

18. Bodansky, H.J., Drury, P.L., Cudworth, A.G. and Evans, D.A.P.: Acetylator phenotype and type I (insulin-dependent) diabetics with microvascular disease. Diabetes 30: 907-910, 1981.

19. Shenfield, G.M., McCann, V.J. and Tjokresetio, R.: Acetylator status and diabetic neuropathy. Diabetologia 22: 441-444, 1982.

20. Pontiroli, A.E., Mosca, A., dePasqua, A., Alcini, D. and Pozza, G.: The fast acetylator phenotype in diabetes mellitus: Abnormal prevalence and association with ABO blood groups. Diabetologia 27: 235-237, 1984.

21. Ladero, J.M., Arrojo, A., deSalamanca, R.E., Gomez, M., Cano, F. and Alfonso, M: Hepatic acetylator phenotype in diabetes mellitus. Ann Clin. Res. 14:187-189, 1982.

22. Miceli, J.N., Olson, W. and Weber, W.: An improved microspectrofluorometic assay for determining isoniazid in serum. Biochem. Med., 12,(4) 348-355, (1975).

23. Olson, W., Miceli, J.N. and Weber, W.: Dose-dependent changes in sulfamethazine pharmacokinetics in rapid and slow INH acetylators. Clin. Pharmacol. Therap., 23, 204-211, (1978).

24. Grant, D.M., Tang, B.K., and Kalow, W.: Polymorphic N-acetylation of a caffeine metabolite. Clin. Pharmacol. Ther. 33:355-359, 1983.

25. Grant, D.M., Campbell, M.E., Tang, B.K. and Kalow, W.: A simple test for acetylator phenotype using caffeine. Brit. J. Clin. Pharmacol. 17:459-464, 1984.

Received: April 6, 1990
Accepted: May 8, 1990