Use of caffeine metabolite ratios to explore CYP1A2 and xanthine oxidase activities

Caffeine was used as a metabolic probe to screen healthy subjects for their activities of two enzymes, deduced to be CYP1A2 (an inducible cytochrome P450) and xanthine oxidase. A longitudinal study revealed modest effects of caffeine dose, ethanol intake, and time-of-day on the CYP1A2 index, without any effect on the xanthine oxidase index. The coefficients of intraindividual variation not accounted for were 5.0% for the xanthine oxidase and 17.2% for the CYP1A2 index. In a population study, both indexes showed a log normal distribution, with CYP1A2 values of most subjects covering a 6.3-fold range but only a 1.7-fold range with xanthine oxidase. The CYP1A2 index was 33% decreased in women who used oral contraceptives and substantially increased in cigarette smokers. Neither the CYP1A2 nor the xanthine oxidase index differed between volunteers of Chinese and European extraction. Four of 178 subjects showed unexplained low xanthine oxidase values (i.e., values several standard deviations below the mean). (CLIN PHARMACOL THER 1991;50:508-19.)

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A systematic exploration of caffeine metabolism both in vivo[1,2] in human subjects and in vitro[3,4] in preparations of human liver microsomes indicated empirically that analyses of caffeine metabolites in urine can yield estimates of the activities of three enzymes[5]: the polymorphic *N*-acetyltransferase,[6,7] xanthine oxidase (XO),[8,9] and CYP1A2, that is one of the cytochromes P450[2,10] that are inducible by polycyclic aromatic hydrocarbons.[11] This article concerns only the latter two enzymes, XO and CYP1A2; the purpose of this article is to define and to explore factors that may cause intraindividual and interindividual variation of the activities of these enzymes as assessed by measuring certain metabolites of caffeine. An extensive report on methods and results of *N*-acetyltransferase determinations, based on the same population study, was published recently.[12] A study of enzyme induction by smoking with use of caffeine as a probe was published separately.[13]

The molecular biology of CYP1A2 has been characterized by Ikeya et al.[14]; it has been shown to activate mutagens[15] and to catalyze the *N*-oxidation of carcinogenic arylamines.[16] Trivial names are P450d in rats, form 4 in rabbits, P_3-450 in mice and humans,[11,17] and phenacetin-*O*-deethylase in rats and humans.[18] Xanthine oxidase can occur in vivo either as an oxygen-dependent oxidase or as a nicotinamide adenine dinucleotide–dependent dehydrogenase[19]; the measurements with caffeine are expected to reveal only the oxidase form.

Caffeine is 1,3,7-trimethylxanthine (137X) and in humans, in contrast to many animals, is mostly metabolized by 3-demethylation to form 1,7-dimethylxanthine (paraxanthine; 17X; Fig. 1).[5,10,20-22] In the tests to be described, the function of caffeine is to serve as the source of 17X. The latter is undergoing two reactions, one of which is 8-hydroxylation by one or more unidentified cytochromes P450 to form 1,7-dimethylurate (17U); the other reaction is 7-demethylation, which leads to two competing products: 1-methylxanthine (1X) and the acetylated ring-split product 5-acetylamino-6-formylamino-3-methyluracil (AFMU). Some portion of 1X is 8-hydroxylated by xanthine oxidase to form 1-methylurate (1U). The extent of AFMU formation is proportional to the activity of the polymorphic *N*-acetyltransferase; AFMU is unstable but it may be quantitatively deformylated to become the stable 5-acetylamino-6-amino-3-methyluracil (AAMU). To assess the 7-demethylation of 17X, one has to consider the sum of AFMU + 1X + 1U or, after stabilizing treatment, of AAMU + 1X + 1U.

Each of the three methyl groups of caffeine is sepa-

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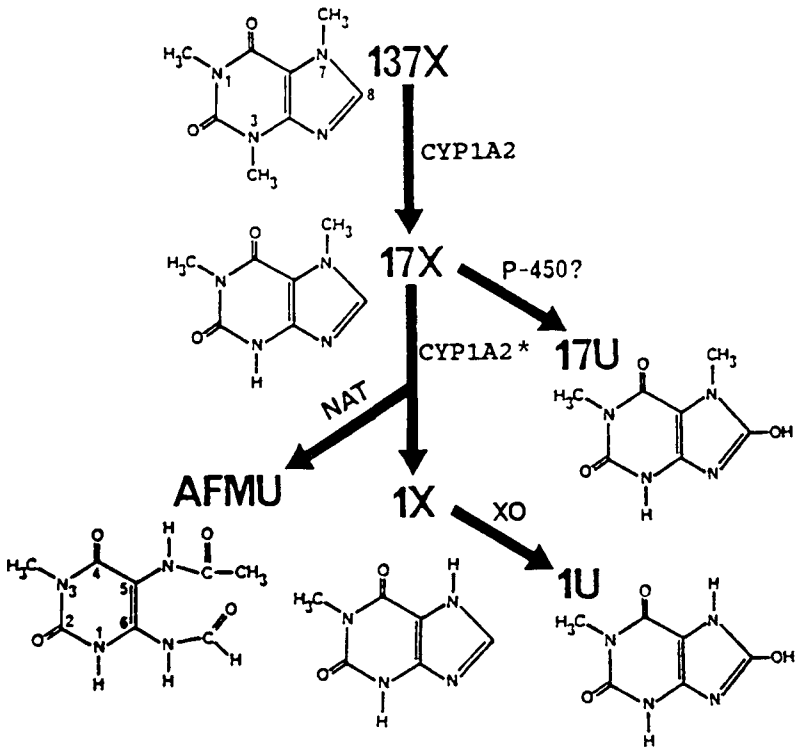

Fig. 1. Caffeine (137X) 3-demethylation and subsequent transformations. The indicated pathway accounts for about 80% of caffeine metabolism, that is, for about 83% of the primary caffeine demethylations. The primary metabolite is paraxanthine (17X). The metabolizing enzymes are CYP1A2 (a cytochrome P450; CYP1A2* identified by indirect means), the polymorphic *N*-acetyltransferase (NAT), and xanthine oxidase (XO). The 8-hydroxylation (1,7-dimethylurate [17U] formation) is attributable to cytochromes P450 that are not fully identified. For testing, the unstable metabolite 5-acetylamino-6-formylamino-3-methyluracil (AFMU) is nonenzymatically converted into the stable 5-acetylamino-6-amino-3-methyluracil (AAMU).

rately subject to demethylation. Fig. 1 shows only the products of the 3-demethylation, that is, paraxanthine (17X) formation, which accounts for 83.9% ± 5.4% of the caffeine demethylations.[22] Only 12.1% ± 4.1% of caffeine is demethylated to theobromine (37X), and none of the secondary metabolites are part of the metabolite ratios of concern in the present context. By contrast, the metabolites 1X and 1U arise from caffeine not only by way of 17X (paraxanthine) but also by way of 13X (theophylline). However, we neglect the theophylline pathway because it usually accounts for only 3.7% ± 1.3% of caffeine demethylation[22] and because much or most of theophylline is metabolized to products other than 1X and 1U.

The caffeine metabolite ratios designating the activities of CYP1A2 and XO may be referred to as CMR_{1A2} and CMR_{XO} or as the CYP1A2 index and XO index, respectively. They are defined here as follows:

$$CMR_{1A2} = \text{CYP1A2 index} = (AAMU + 1X + 1U)/17U$$
$$CMR_{XO} = \text{XO index} = 1U/(1X + 1U)$$

Measurements of acetylation capacity are not a topic of this article. The *N*-acetyltransferase index can be assessed as the ratio AAMU/(AAMU + 1X + 1U) or, when simplification is appropriate or desirable, as AAMU/1X or AFMU/1X.[12]

It has been established by Butler et al.[18] that the caffeine 3-demethylation, the main route of caffeine metabolism in humans,[5,10,20-22] is catalyzed by cytochrome CYP1A2. Berthou et al.[23] recently confirmed this observation. Previous studies that used liver microsomes from 15 kidney donors[3] showed that ethoxyresorufin *O*-deethylation correlated with caffeine

3-demethylation ($r = 0.96$) and with paraxanthine 7-demethylation ($r = 0.94$) and that both demethylations correlated with each other ($r = 0.83$).[3] Ethoxyresorufin is *O*-deethylated in human liver by CYP1A2.[24] It was therefore concluded that CYP1A2 in humans must also be mainly responsible for the 7-demethylation of paraxanthine (see enzyme designations in Fig. 1).

In vitro, all xanthine demethylation reactions are potentially catalyzed by more than one enzyme in most livers.[3,4,23] Hence, support by in vivo studies is required for the conclusion that the 7-demethylation of paraxanthine must be catalyzed mostly or completely by the main caffeine-metabolizing enzyme.

Campbell et al.[2] have shown empirically that caffeine clearance correlates significantly ($r = 0.91$) with the CYP1A2 metabolic ratio (1X + 1U + AAMU)/17U in urine; this correlation validates the interpretation of the ratio and thereby confirms the previous conclusion[1] that the metabolite appearance in urine reflects metabolite formation rather than metabolite renal clearance (this statement does not apply to 17X, which is therefore not part of the equations). The validity of this correlation was emphasized by the observation of Campbell et al.[2] (confirmed here with a larger number) that the metabolic ratio is decreased in women taking oral contraceptives and increased in smokers, changes that are known to occur from independent measurements of metabolic clearances of caffeine.[25-27] A comparison of the CYP1A2 index with the results of the caffeine breath tests in 44 adults gave a correlation coefficient of $r = 0.74$[28]; the caffeine breath test is designed to assess the 3-demethylation of caffeine by way of the exhalation of labeled carbon dioxide.

Studies in vitro indicate that xanthine oxidase is the enzyme causing the 8-hydroxylation of 1X, thus forming 1U.[8,9] In support of this observation, in vivo studies of several subjects have shown that the XO index is decreased after intake of the XO inhibitor allopurinol.[8] Furthermore, this ratio correlates with one reflecting endogenous compounds, namely, the ratio (xanthine plus hypoxanthine)/urate.[8] If 1X is formed from theophylline after theophylline administration, virtually all is converted into 1U; the reason for the incomplete oxidation of 1X after caffeine administration is not clear.[1]

METHODS

The findings reported in this article were obtained by two independently conducted studies: (1) a longitudinal study of 11 healthy male volunteers over several months with systematic variation of caffeine dose and lifestyle factors and (2) a population study, that is, a single-dose study of 178 healthy student volunteers.

The programs and the subjects of investigation

The longitudinal study. Eleven healthy, adult white men between the ages of 22 and 29 years (mean age, 26.3 years) took part in this study. Except for one subject, their average weights were (mean ± SD) 77.3 ± 6.4 kg and their average coffee consumption was 3.4 ± 0.7 cups per day; one subject (No. 3) of exceptional height (196 cm) and weight (105 kg) regularly consumed 8 cups. Only one subject (No. 7) was a regular smoker (10 cigarettes a day).

As stipulated by the Human Experimentation Committee of the University of Toronto, only men were allowed to volunteer for this study. The only other exclusion criteria were disease, long-term use of medication, and allergies. Volunteers were recruited through pamphlets distributed in the laboratories of the Department of Pharmacology. The majority of participants were graduate students; all participants were well aware of the purpose of the study and the need for reliability in collecting the urine samples and measuring the volumes. Eleven subjects were recruited and all completed the study.

There were four study periods. There was a minimum of 7 days between each study period, during which the subjects resumed their usual lifestyles. During the study periods, the subjects did not take drugs or consume chocolates.

Study 1: lifestyle. During 10 days, the subjects collected all of their urine, divided into day (8 AM to midnight) and night (midnight to 8 AM) specimens. Each subject accumulated and measured his volumes of the day and of the night and brought daily a 10 ml aliquot of each into the laboratory with the pH of each sample adjusted to about 3.5 by the addition of 20 mg/ml ascorbic acid. There the specimens were stored at −20° C until the time of analysis. The volunteers were encouraged to continue their accustomed lifestyles with the qualification that each should consume between one and four cups of coffee of his choice per day; for one subject, this meant a restriction of his habitual coffee intake, whereas it meant intake as usual for all other subjects.

For the following three studies, coffee was supplied as a commercial freeze-dried preparation to yield 75 mg caffeine per cup. Except for the dose variation study, the subjects were instructed to consume two (and only two) of the standardized cups per day during

the study periods. The time of consumption was not specified.

Study 2: dose variation. For a period of 8 days the daily caffeine intake was systematically altered as follows: days 1 and 2, 1 cup per day; days 3 and 4, 2 cups per day; days 5 and 6, 4 cups per day; and days 7 and 8, 1 cup per day. In this study, as in the following studies, only the night urine was collected, which included the first void of the morning.

Study 3: multivitamin intake. On days 1 through 8, one multivitamin tablet was consumed. The subjects pursued their accustomed lifestyles as in study 1 except for the intake of standardized coffee.

Study 4: ethanol intake. Each drink contained 12 gm ethanol; that is, it consisted of 1 ounce of 40% ethanol solution diluted with orange juice as individually desired. Provisions were made to ensure the subjects' safety on all test days. The ethanol beverage was consumed as follows: days 1 and 2, 1 drink per day; days 3 and 4, 2 drinks per day; days 5 and 6, 4 drinks per day; and days 7 and 8, 1 drink per day. The time of drinking on each day was not specified, but the drinks were to be consumed at the rate of about one drink per hour.

The population study. Posters calling for volunteers for this study were displayed in the Medical Sciences Building of the University of Toronto; hence the volunteers included members of the professional and technical staff. However, most of the volunteers were students. All stated that they were in good health and did not suffer from allergies. Any long-term use of medication was an exclusion criterion. They were asked about their smoking habits and their intakes of any medication. They were requested to refrain from chocolate, alcohol, and drug consumption for one day before the test. In the afternoon of the test day, they were asked to consume at least one but not more than four cups of coffee or a strong tea or at least two caffeine-containing soft drinks. They were asked to bring their first-void (morning) urine of the following day to the laboratory. The study was approved by the Human Experimentation Committee of the University of Toronto.

One hundred eighty-six subjects were recruited but only 178 completed the test; lack of completion meant, in most cases, inadvertent consumption of only decaffeinated coffee. Useful data were obtained from 106 men and 72 women. Average age was 25.5 ± 7.8 years (mean ± SD), body weight was 68.2 ± 11.6 kg, and height was 174 ± 10 cm. They habitually consumed 2.0 ± 1.4 cups of coffee per day and took 0.5 ± 0.6 alcoholic drinks; only 19 called themselves smokers but most of these were light smokers (see Kalow and Tang[13] for the separate report on this aspect of the study). Eighteen of the women used oral contraceptives. Of the volunteers, 144 were white, 21 were Orientals, and 13 had other racial backgrounds.

Laboratory procedures

The xanthines and urates were first extracted by organic solvents and then determined by HPLC as described previously.[1] After complete conversion of AFMU into AAMU at pH 10, the AAMU was measured by exclusion chromatography as described previously.[29] Potential pitfalls in the measurement of caffeine metabolites were described in a recent publication.[12]

Creatinine was determined as described by Faulkner and King[30]; D-glucaric acid was determined as described by Colombi et al.[31]

For statistical analysis of the longitudinal study, the ANOVA was of a split-plot design and was performed by use of the statistical application program SYSTAT (Systat Inc., Evanston, Ill.). All other statistical analyses were routine procedures.

RESULTS

Reliability of the measured parameters

The findings of the longitudinal study for each individual are indicated in Tables I and II and in Fig. 2 and 3. It is apparent that the differences between subjects are considerably larger for the CYP1A2 (Table I) than for the XO index (Table II). The subjects with the highest and lowest values tend to keep their ranks. Subject 7 was the only smoker in this group; he claimed to smoke 10 cigarettes per day and his CYP1A2 index is conspicuous (Table I).

Tables I and II show between-subject but not within-subject variation in any given period. Figs. 2 and 3 show both between- and within-subject variation for the study periods designated "dose" and "ethanol" but only with respect to the CYP1A2 index. Not presented are the within-subject CYP1A2 data for the three periods designated "day," "night," and "vitamins" or any of the within-subject XO data. However, all 484 measurements were used in an ANOVA in a search for significant factors that may influence the magnitude or variation of either the CYP1A2 or the XO index. Following is a brief summary of the significant factors that were discovered.

The longitudinal study revealed effects of caffeine dose ($p < 0.001$; Fig. 1), of ethanol intake ($p < 0.01$; Fig. 2), and of time of day ($p < 0.01$; Table I) on the CYP1A2 index with no effect on the XO index. Halv-

Table I. Longitudinal study: the CYP1A2 index

Subject No.	*Dose*	*Vitamin*	*Ethanol*	*Night*	*Day*
1	6.7	6.9	7.1	7.5	6.6
2	7.7	5.8	7.7	7.4	6.6
3	7.0	7.0	5.6	6.7	5.4
4	6.7	6.1	5.8	4.6	4.5
5	8.3	7.1	7.9	8.9	7.1
6	8.7	7.5	7.1	7.0	7.0
7	11.3	12.2	12.7	11.0	8.0
8	6.9	6.8	6.5	6.3	6.3
9	6.4	5.6	6.7	6.8	6.7
10	6.2	5.9	4.9	5.1	4.8
11	3.9	4.5	4.7	4.6	4.1
Mean ± SD	7.3 ± 1.8	6.9 ± 1.9	7.0 ± 2.1	6.9 ± 1.8	6.0 ± 1.1

Each entry represents the average for a given subject during a study period.

Table II. Longitudinal study: the xanthine oxidase index

Subject No.	*Dose*	*Vitamin*	*Ethanol*	*Night*	*Day*
1	0.53	0.58	0.56	0.61	0.56
2	0.65	0.59	0.61	0.66	0.63
3	0.59	0.60	0.60	0.65	0.63
4	0.59	0.67	0.60	0.62	0.64
5	0.64	0.65	0.65	0.65	0.63
6	0.52	0.58	0.56	0.59	0.58
7	0.64	0.69	0.68	0.65	0.62
8	0.64	0.66	0.66	0.67	0.69
9	0.63	0.64	0.69	0.68	0.68
10	0.53	0.60	0.61	0.54	0.58
11	0.57	0.59	0.59	0.60	0.59
Mean ± SD	0.59 ± 0.03	0.62 ± 0.03	0.62 ± 0.03	0.63 ± 0.03	0.62 ± 0.02

Each entry represents the average for a given subject during a study period.

ing the standardized daily intake of two cups of coffee (150 mg caffeine) increased the mean CYP1A2 index by 10%, doubling reduced it by 15%. There was evidence of a circadian rhythm (daytime, 87% of nighttime average) with less intrasubject variability during the day than during the night. The short-term consumption of four drinks (48 gm ethanol) elevated the CYP1A2 index by 12%. The coefficients of unaccounted-for intraindividual variation as during the "vitamin" period were 5.0% for the XO index and 17.2% for the CYP1A2 index.

Further scrutiny of the dose study reveals that the CYP1A2 index averaged the lowest values on the two "four-cup" days (6.08 ± 0.54 [mean ± SEM]) and highest values (7.80 ± 0.61) on the four "one-cup" days (Fig. 2); it is these latter values that cause the elevation registered in Table I. Because the CYP1A2 index represents the metabolite ratio (AAMU + 1X + 1U)/17U, the numerator and denominator were separately analyzed; this revealed that the increase from one to four cups caused a 3.5-fold increase of the denominator but an only threefold increase of the numerator. Thus, with 4 cups of coffee (300 mg caffeine), a saturation effect of the enzymatic capacity for 7-demethylation of paraxanthine is showing in the majority of subjects. This analysis also indicates that a major function of the denominator in this equation is to correct for caffeine dose variation.

The similarities between the entries marked night and vitamin deserve notice (Tables I and II). The vitamin intake in this context served as a placebo. During the vitamin study, the subjects were given 150 mg of caffeine daily for consumption as coffee, whereas during the night study, the subjects consumed their own coffee as they wished between the limits of 1 to 4 cups per day.

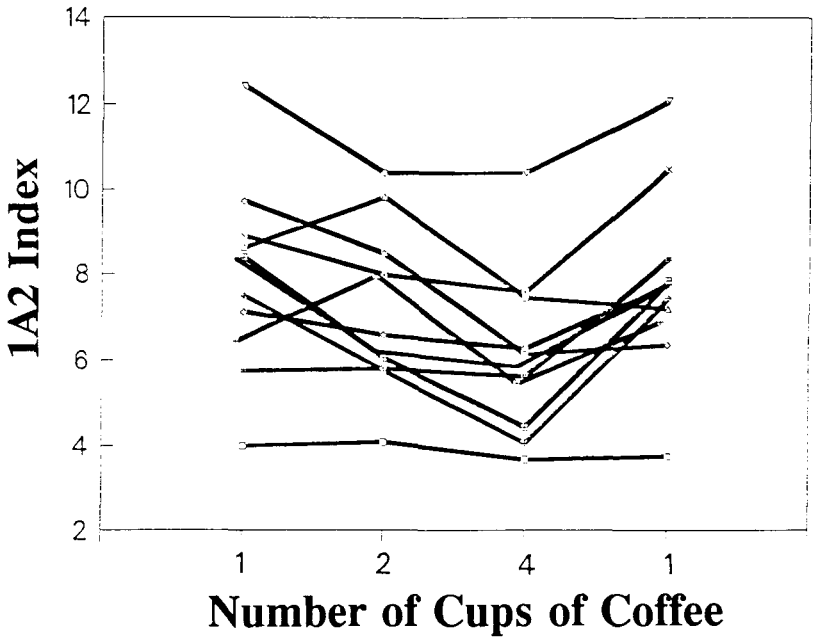

Fig. 2. Effects of varying coffee intake on the CYP1A2 index in 11 subjects (one cup = 75 mg caffeine). Each value is the mean of measurements on 2 consecutive days during an experimental period of 8 days. The caffeine dose-dependent variation is significant ($p < 0.001$), as established by ANOVA, comparing the CYP1A2 fluctuations in all five experimental periods.

The XO index was essentially unchanged under all of the tested conditions (Table II).

Variability and interdependence of the measured parameters

A summary of the measurements on the urine specimens from 178 volunteers is indicated in Table III. There are three groups of data:

The first two rows represent baseline data; namely, measurements of creatinine concentration in the test samples of urine and of glucaric acid concentration per creatinine concentration, an entity that serves as an indicator of exposure to barbiturate-type inducers.[31] The next five rows indicate the concentrations of caffeine metabolites in the investigated samples of urine. The last two rows indicate the CYP1A2 and the XO indexes. The characteristics of the population distribution of each measurement are given in statistical terms. The following observations deserve comment.

As indicated in the Methods section, the dose of caffeine taken by each volunteer was allowed to vary within loose limits; no attempt was made to actually measure the dose. Hence, the coefficient of variation (the SD as a percentage of the mean) of the excretion of caffeine metabolites must reflect to a large extent the dose variation. The total amount of metabolites excreted is the least variable (coefficient of variation, 73.6%) of these items. The coefficients of variation of most individual metabolites exceed 90%, which must reflect person-to-person differences in addition to the dose variation. The indexes of cytochromal and XO activities vary much less than does the excretion of individual metabolites; formation of the ratios that make up these indexes has grossly diminished or eliminated the effects of varying doses. There is a low coefficient of variation for the XO index (12.4%) compared with the CYP1A2 index (40.6%).

As shown by Table III, distribution curves of all five metabolite measurements and that of the CYP1A2 index are skewed; they can all be normalized by logarithmic transformation of the data (e.g., the log normal distribution of the CYP1A2 index in Fig. 4). All calculations involving these data (e.g., as search for differences between subpopulations) were conducted with use of both raw and logarithmic data. Because the findings were not different, only the raw data were tabulated for the sake of simplicity. The raw data on creatinine and glucaric acid appeared to be normally distributed.

The metabolic ratios representing the XO index yielded similar distribution curves whether plotted raw

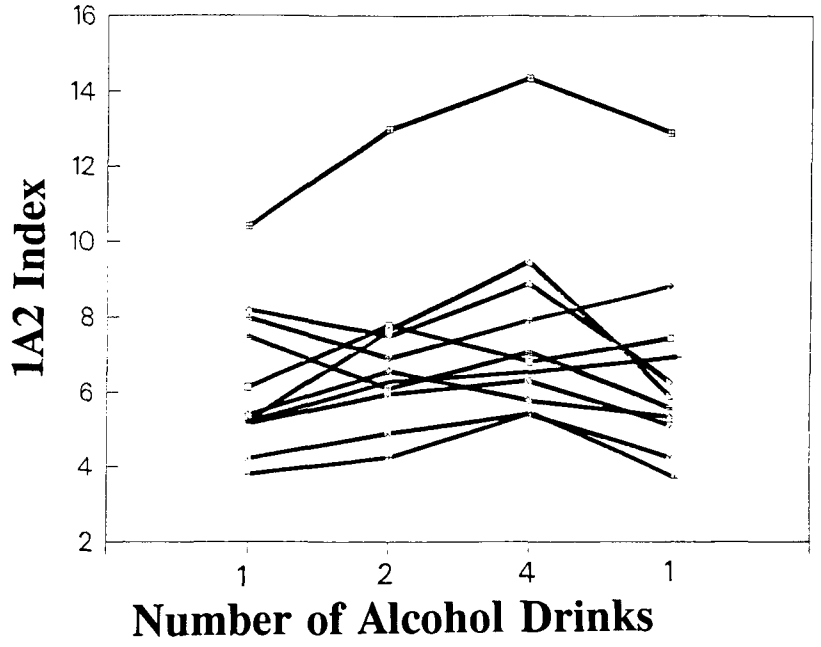

Fig. 3. Effect of ethanol intake on the CYP1A2 index in 11 subjects. (one drink = 12 gm ethanol). Each value is the mean of the 2 days measurements. Comparative analysis (see text and legend to Fig. 2) showed the ethanol effect to be significant with $p < 0.01$.

Table III. Population study of caffeine metabolism

	Mean ± SE	*Coefficient of variation (%)*	*Skewness*	*Kurtosis*
Creatinine (gm/L)	1.83 ± 0.05	39.9	0.17	2.5
Glucaric acid (μmol/gm C)	21.92 ± 0.89	54.1	0.67	4.2
1U (mmol/L)	0.166 ± 0.012	92.6	2.6	14.1
1X (mmol/L)	0.128 ± 0.009	90.6	2.3	11.4
17U (mmol/L)	0.087 ± 0.006	91.4	2.3	11.2
AAMU (mmol/L)	0.141 ± 0.008	77.4	1.4	4.8
Total (mmol/L)	0.556 ± 0.031	73.6	1.6	7.4
CYP1A2 Index*	5.96 ± 0.18	40.6	1.05	4.1
XO Index†	0.57 ± 0.01	12.4	−0.60	4.3

Data derived from measurements in urine.
1U, 1-Methylurate; 1X, 1-methylxanthine; 17U, 1,7-dimethylurate; AAMU, 5-acetyl-6-amino-3-methyluracil; C, creatinine.
*CYP1A2 index = (1U + 1X + AAMU)/17U.
†XO index = 1U/(1U + 1X).

or logarithmically; Fig. 5 shows a logarithmic plot. A striking feature is the occurrence of very low values in a few subjects; the abscissa is scaled in standard deviations from the mean to indicate that the values are outside the range of normal variability. The subjects had a normal CYP1A2 index and they included slow and fast acetylators. There was no evidence of alcohol abuse or other intoxication.

A correlation matrix between the data summarized in Table III is shown in Table IV. This table yields further insights:

- The concentration of creatinine shows low but significant levels of correlation with the concentration of every metabolite in that sample, pointing to urinary water as the common variable. This observation validates the use of creatinine as a basis for quoting relative amounts of excreted substances.
- The excretion of glucaric acid shows a statistically

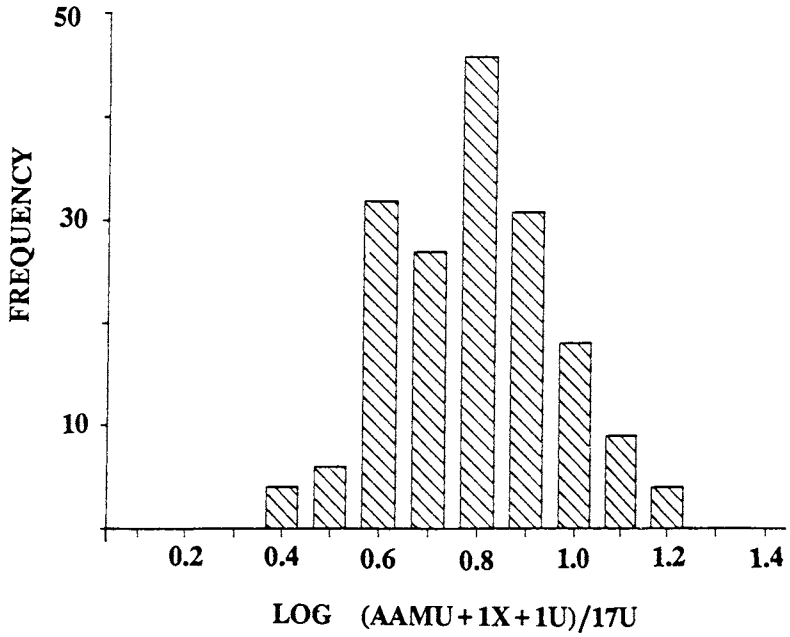

Fig. 4. Frequency distribution of the log CYP1A2 index in 178 healthy subjects.

significant but a biologically only marginal correlation with the excretion rates of 1U, 1X, and the metabolite total. In other words, some influence of barbiturate-type inducers on the metabolite pattern cannot be excluded, but it is not noticeably reflected in the enzyme indexes.

- The total amount of metabolites correlates strongly with the excretion of the components that make up this total. However, the total amount excreted is unrelated to XO activity and only marginally correlated with the CYP1A2 index.
- The only item in Table IV that shows a correlation with every other item is the excretion of 1-methylxanthine. The reason or the reasons for this special status are not clear.
- The measurement of 17U is a relatively strong determinant of the CYP1A2 index. The correlation is negative since 17U is the denominator in the ratio used.
- The identity of the cytochromes which catalyze the formation of 17U by hydroxylating paraxanthine is not fully known. Hence it deserves to be noted that 17U excretion does not correlate with the presence of glucaric acid.
- The high correlation of 17U excretion with that of 1-methylxanthine and 1U can be assumed to reflect mainly the fact that the concentration of these products has as a joint determinant the concentration of paraxanthine as the parent compound from which they all derive.[10] The correlation between 17U and AAMU is less than that of the other products arising from paraxanthine because the proportion of AAMU also depends on *N*-acetyltransferase activity.

Differences and similarities between population subgroups

The search for differences within the studied population led to the following observations. There was no significant correlation of body weight, age, or customary caffeine consumption with either the CYP1A2 or the XO index. There was an influence of gender upon the CYP1A2 index that averaged 6.36 ± 0.24 (mean ± SEM) for men and 5.36 ± 0.27 for women. The difference was significant at $p < 0.05$; however, this difference is deceptive. If the group of 72 women is subdivided into 18 users and 54 nonusers of oral contraceptives, the respective values are 3.90 ± 0.32 and 5.85 ± 0.31; in short, there is no principal gender difference of the CYP1A2 index (6.36 versus 5.85, not significant) but the use of oral contraceptives causes a substantial decrease ($p < 0.001$).

Several subjects called themselves smokers but smoked only 1 or 2 cigarettes per day. Of the 19 self-designated smokers, 8 smoked 10 or more cigarettes daily; their CYP1A2 index was 8.52 ± 1.04 ($p < 0.02$). A further analysis of the relationships

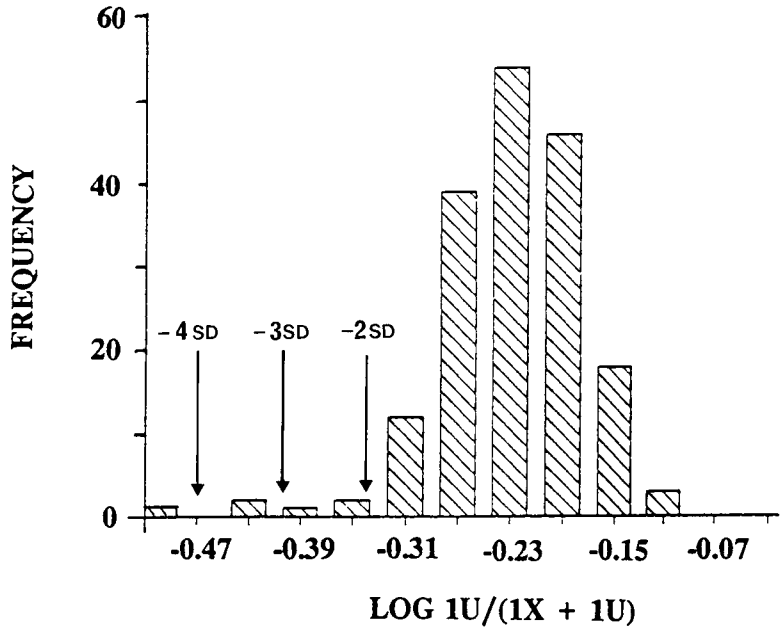

Fig. 5. Frequency distribution of the log XO index in 178 healthy subjects. The *arrows* mark SDs −2, −3, and −4, respectively, as an indication that at least three of the low values (more likely four) are outside expectation.

Table IV. Population study of caffeine metabolism: Spearman rank correlation matrix

GlA	−0.01							
1U	0.20*	0.22*						
1X	0.27†	0.20*	0.94†					
17U	0.35†	0.14	0.85†	0.86†				
AAMU	0.25†	0.14	0.62†	0.54†	0.69†			
Total	0.27†	0.20*	0.94†	0.92†	0.93†	0.79†		
CYP1A2	−0.23*	0.09	−0.11	−0.19*	−0.51†	−0.03	−0.21*	
XO	−0.25†	0.09	0.05	−0.24†	−0.14	0.16	−0.03	−0.29†
	C	GlA	1U	1X	17U	AAMU	Total	CYP1A2

GlA, Glucaric acid; 1U, 1-methylurate; 1X, 1-methylxanthine; 17U, 1,7-dimethylurate; AAMU, 5-acetyl-6-amino-3-methyluracil; CYP1A2, CYP1A2 index [(1U + 1X + AAMU)/17U]; XO, XO index [1U/(1U + 1X)].
*$p < 0.01$.
†$p < 0.001$.

between smoking, cotinine excretion, and the CYP1A2 index in this population has been published separately.[13]

Comparisons of the data derived from the Oriental subjects and white subjects in the studied population showed the same trends that were observed previously.[5] That is, neither the CYP1A2 index nor the XO index differed between the racial groups, although individual metabolites differed. 1X and 1U were significantly lower and AAMU was higher in Oriental subjects, which is consistent with the higher proportion of rapid acetylators in Oriental compared with white populations.[32]

DISCUSSION

Some comparisons with literature data

In a recent study, Kadlubar et al.[33] used a ratio of paraxanthine and caffeine in urine to assess CYP1A2 activity in 30 subjects; the composition of this population was not defined, so it is not possible to compare results. We have formerly measured paraxanthine in urine[1] but gave it up because of variability in its renal elimination; we suspect that individual and ethnic variability of tubular transport[5] as factors that remain to be analyzed. Furthermore, paraxanthine, like caffeine itself, is excreted as a minor metabolite because of its metabolic degradation. Nevertheless, there is

room for a systematic comparison of methods, particularly in subjects with enzymes defined by liver biopsy. In vivo assessment of drug metabolism does not readily yield a precise measure of the activity of any enzyme; however, to be useful the assessment must be able to provide relative measures that reveal enzyme variation.

In our hands, the population distribution of the measurements reflecting CYP1A2 activity was log normal; we have to conclude that the enzyme is not polymorphically (i.e., frequently) variable in white subjects. Many years ago this enzyme was shown to be variable within a family in which the deficiency was noticed by the absence of phenacetin-*O*-deethylation.[34] It thus appears that there are genetic variants of this cytochrome but that they are too rare to have shown up in a population sample of the size investigated here.

As shown previously,[1] XO activity as assessed through caffeine metabolism is remarkably uniform in healthy white and Oriental subjects. The occurrence of distinctly low activity in four of 178 subjects is a surprise that requires further study. There are on record very rare cases of genetic deficiency of XO[35]; by definition, heterozygous carriers should be less rare but probably not in the order of 2% of a population. Environmental influences are conceivable because molybdenum cofactor is necessary, at least for the xanthine dehydrogenase activity of xanthine oxidase.[36]

Use of caffeine as a metabolic probe

As recently emphasized by Cheng et al.[37] and as shown above in the longitudinal study, the caffeine demethylation rate is saturable. If the purpose of measuring the CYP1A2 index is clinical assessment of the liver function of a given patient, the adherence to a fixed caffeine dose and a precisely timed intake must be recommended.

However, optimal testing conditions for clinical and for epidemiologic purposes are not necessarily identical—ethical, personal, economic, and informational requirements are different. Our past recommendation to limit the caffeine intake on the test day to not less than 1 cup and to not more than 4 cups seems to be adequate if the purpose is a population study of the CYP1A2 and the XO indexes (or of the acetylation polymorphism[12]). These restrictions reduce the problem of enzyme saturation to acceptable limits, as suggested by the similarity of the vitamin and the night data in the longitudinal study; further, the index variation accountable by dose variation within the stated limits is small compared with the range of values encountered in a population or between smokers and nonsmokers. Attention to the circadian variation of the CYP1A2 index may be more important than the effects of dose variation within reasonable limits. Furthermore, caffeine demethylation rates or caffeine half-lives are reduced in liver disease.[21] Also, there are various drugs capable of inhibiting caffeine metabolism.[38]

The large unaccounted-for intraindividual variation of the CYP1A2 index is probably biologic reality; Shively and Vesell[39] described in 1975 large temporal intraindividual variations of phenacetin half-life that may be another indication of the variability of the same enzyme.

The same caffeine assays that yield the CYP1A2 and the XO indexes may also be analyzed to yield information on the activity of the genetically variable *N*-acetyltransferase.[6,7,12,40,41] Assessments of the activities of each of the three enzymes that can be assayed with caffeine are of pharmacologic interest and of potential significance for cancer epidemiology. The genetically low activity of *N*-acetyltransferase has been shown to be associated with some drug toxicities and with the occurrence of arylamine-produced bladder cancers.[32,42] CYP1A2 catalyzes the *N*-oxidation of numerous arylamines; thus, like CYP1A1 (the aryl-hydrocarbon hydroxylase), CYP1A2 converts precarcinogens into carcinogens.[16,24,43] CYP1A2 and CYP2E1 are the enzymes that convert excess acetaminophen into a hepatotoxin.[44] XO activity is a prominent source of active oxygen species in the mammalian body[19,45] with established importance for reperfusion injury[46] and potential significance for the mechanism of aging[47] and for carcinogenicity[48]; it is inducible by interferon.[49]

In conclusion, analysis of one sample of urine after intake of a caffeine-containing beverage may serve as an empiric means to determine, simultaneously and noninvasively, the activities of an inducible cytochrome P450 and of XO. It is not surprising that there are factors that may distort the caffeine-derived information. However, if used judiciously, the enzyme assays with caffeine are able to reveal intersubject and intrasubject variability and thereby provide a means to investigate the factors causing such variability. A control of caffeine dosage is appropriate for clinical studies, but dose variation within stated limits seems to be tolerable in epidemiologic studies. As described before, the same measurements of the same caffeine metabolites but as components of a different equation may be used to determine the phenotype of the genetically variable *N*-acetyltransferase.

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References

1. Grant DM, Tang BK, Kalow W. Variability in caffeine metabolism. CLIN PHARMACOL THER 1983;33:591-602.
2. Campbell ME, Spielberg SP, Kalow W. A urinary metabolite ratio that reflects systemic caffeine clearance. CLIN PHARMACOL THER 1987;42:157-65.
3. Campbell ME, Grant DM, Inaba T, Kalow W. Biotransformation of caffeine, paraxanthine, theophylline, and theobromine by polycyclic aromatic hydrocarbon-inducible cytochrome(s) P-450 in human liver microsomes. Drug Metab Dispos 1987;15:237-49.
4. Grant DM, Campbell ME, Tang BK, Kalow W. Biotransformation of caffeine by microsomes from human livers. Kinetics and inhibition studies. Biochem Pharmacol 1987;36:1251-60.
5. Kalow W. Variability of caffeine metabolism in humans. Arzneimittelforschung 1985;35:319-24.
6. Grant DM, Tang BK, Kalow W. A simple test for acetylator phenotype using caffeine. Br J Clin Pharmacol 1984;17:459-64.
7. Tang BK, Kadar D, Kalow W. An alternative test for acetylator phenotyping with caffeine. CLIN PHARMACOL THER 1987;42:509-13.
8. Grant DM, Tang BK, Campbell ME, Kalow W. Effect of allopurinol on caffeine disposition in man. Br J Clin Pharmacol 1986;21:454-8.
9. Boda D, Nemeth I. Measurement of urinary caffeine metabolites reflecting the "in vivo" xanthine oxidase activity in premature infants with RDS and in hypoxic states of children. Biomed Biochim Acta 1989; 48:S31-5.
10. Kalow W, Campbell M. Biotransformation of caffeine by microsomes. ISI Atlas of Science 1988;2:381-6.
11. Okey AB. Enzyme induction in the cytochrome P-450 system. Pharmacol Ther 1990;45:241-98.
12. Tang BK, Kadar D, Qian L, Iriah J, Yip J, Kalow W. Caffeine as a metabolic probe: validation of its use for acetylator phenotyping. CLIN PHARMACOL THER 1991; 49:648-57.
13. Kalow W, Tang BK. Caffeine as a metabolic probe: exploration of the enzyme-inducing effect of cigarette smoking. CLIN PHARMACOL THER 1991;49:44-48.
14. Ikeya K, Jaiswal AK, Owens RA, Jones JE, Nebert DW, Kimura S. Human CYP1A2: sequence, gene structure, comparison with the mouse and rat homologous gene, and differences in liver mRNA expression. Mol Endocrinol 1989;3:1399-408.
15. Aoyama T, Gonzalez F, Gelboin HV. Human cDNA-expressed cytochrome P450 1A2: mutagen activation and substrate specificity. Mol Carcinog 1989;2:192-8.
16. Guengerich FP, Shimada T, Iwasaki M, Martin MV. Activation of mutagens by human cytochrome P-450 enzymes. In: Mendelsohn ML, Albertini RJ, eds. Mutation and the environment. Part B: metabolism, testing methods, and chromosomes. New York: John Wiley & Sons, 1990:87-96.
17. Gonzalez FJ. Molecular genetics of the P-450 superfamily. Pharmacol Ther 1990;45:1-38.
18. Butler MA, Iwasaki M, Guengerich FP, Kadlubar FF. Human cytochrome $P\text{-}450_{PA}$ (P-450IA2), the phenacetin-*O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and *N*-oxidation of carcinogenic arylamines. Proc Natl Acad Sci USA 1989;86:7696-700.
19. Parks DA, Granger DN. Xanthine oxidase: biochemistry, distribution and physiology. Acta Physiol Scand 1986;348(suppl):87-99.
20. Arnaud MJ. Products of metabolism of caffeine. In: Dews PE, ed. Caffeine. New York: Springer, 1984:1-38.
21. Bonati M, Garattini S. Pharmacokinetics of caffeine. ISI Atlas of Science 1988;2:33-9.
22. Lelo A, Miners JO, Robson RA, Birkett DJ. Quantitative assessment of caffeine partial clearances in man. CLIN PHARMACOL THER 1986;22:183-6.
23. Berthou F, Flinois JP, Ratanasavanh D, Beaune P, Riche C, Guillouzo A. Evidence for the involvement of several cytochromes P-450 in the first steps of caffeine metabolism by human liver microsomes. Drug Metab Dispos 1990;19:561-7.
24. Gonzalez FJ, Aoyama T, Gelboin HV. Activation of promutagens by human cDNA-expressed cytochrome P450s. In: Mendelsohn ML, Albertini RJ, eds. Mutation and the environment. Part B: metabolism, testing methods, and chromosomes. New York: John Wiley & Sons, 1990:77-86.
25. Callahan MM, Robertson RS, Branfman AR, McComish MF, Yesair DW. Comparison of caffeine metabolism in three non smoking populations after oral administration of radiolabeled caffeine. Drug Metab Dispos 1983;11:211-7.
26. Parsons WD, Neims AH. Effect of smoking on caffeine clearance. CLIN PHARMACOL THER 1978;24:40-5.
27. Callahan MM, Robertson RS, Arnaud MJ, Branfman AR, McComish MF, Yesair DW. Human metabolism of [1-methyl-^{14}C]- and [2-^{14}C]caffeine after oral administration. Drug Metab Dispos 1982;10:417-23.
28. Lambert GH, Schoeller DA, Humphrey HEB, et al. The caffeine breath test and caffeine urinary metabolite ratios in the Michigan cohort exposed to polybrominated biphenyls: a preliminary study. Environ Health Perspect 1990;89:175-81.
29. Tang BK, Zubovits T, Kalow W. Determination of acetylated caffeine metabolites by high-performance exclusion chromatography. J Chromatog 1986;375:170-3.
30. Faulkner WR, King JW. Renal function. In: Tietz NW, ed. Fundamentals of clinical chemistry. Philadelphia: WB Saunders, 1970:997.
31. Colombi A, Maroni M, Antonini C, Cassina T, Gambini A, Foa V. Low-pH method for the enzymatic assay of D-glucaric acid in urine. Clin Chim Acta 1983;128:337-47.

32. Evans DAP. *N*-Acetyltransferase. Pharmacol Ther 1989;42:157-234.
33. Kadlubar FF, Talaska G, Butler MA, Teitel CH, Hassengill JP, Lang NP. Determination of carcinogenic arylamine *N*-oxidation phenotype in humans by analysis of caffeine urinary metabolites. In: Mendelsohn ML, Albertini RJ, eds. Mutation and the environment. Part B: Metabolism, testing methods, and chromosomes. New York: John Wiley & Sons, 1990:107-14.
34. Shahidi NT. Acetophenetidin-induced methemoglobinemia. Ann NY Acad Sci 1968:151:822-32.
35. Holmes EW, Wyngaarden JB. Hereditary xanthinuria. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic basis of inherited disease. New York: McGraw-Hill, 1989:1085-94.
36. Johnson JL, Wadman WK. Molybdenum cofactor deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic basis of inherited disease. New York: McGraw-Hill, 1989:1463-75.
37. Cheng WS, Murphy TL, Smith MT, Cooksley GE, Halliday JW, Powell LW. Dose-dependent pharmacokinetics of caffeine in humans: relevance as a test of quantitative liver function. Clin Pharmacol Ther 1990;47:516-24.
38. Tarrus E, Cami J, Roberts DJ, Spickett RGW, Celdran E, Segura J. Accumulation of caffeine in healthy volunteers treated with furafylline. Br J Clin Pharmacol 1987;23:9-18.
39. Shively CA, Vesell ES. Temporal variations in acetaminophen and phenacetin half-life in man. Clin Pharmacol Ther 1975;18:413-24.
40. Hildebrand M, Seifert W. Determination of acetylator phenotype in Caucasians with caffeine. Eur J Clin Pharmacol 1989;37:525-6.
41. Kilbane AJ, Silbart LK, Manis M, Beitins IZ, Weber WW. Human *N*-acetylation genotype determination with urinary caffeine metabolites. Clin Pharmacol Ther 1990;47:470-6.
42. Weber WW. The acetylator genes and drug response. New York: Oxford University Press, 1987.
43. Shimada T, Iwasaki M, Martin MW, Guengerich FP. Human liver microsomal cytochrome P-450 enzymes involved in the bioactivation of procarcinogens detected by umu gene response in Salmonella typhimurium TA 1535/pSK1002. Cancer Res 1989;49:3218-28.
44. Raucy JL, Lasker Jm, Lieber CS, Black M. Acetaminophen activation by human liver cytochromes P450IIEI and P450IA2. Arch Biochem Biophys 1989;271:270-83.
45. Byczkowski JZ, Gessner T. Action of xanthine-xanthine oxidase system on microsomal benzo(a)pyrene metabolism in vitro. Gen Pharmacol 1987;18:385-95.
46. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 1985;312:159-63.
47. Schoutsen B, Willem de Jong J. Age-dependent increase in xanthine oxidoreductase differs in various heart cell types. Circ Res 1987;61:604-7.
48. Ames BN. Measuring oxidative damage in humans: relation to cancer and ageing. In: Bartsch H, Hemminki K, O'Neill IK, eds. Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention. IARC Sci Publ 1988;89:407-16.
49. Renton KW, Knickle LC. Regulation of hepatic cytochrome P450 during infectious disease. Can J Physiol Pharmacol 1990;68:777-81.