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# Assay of caffeine metabolism *in vitro* by human liver microsomes using radio-high-performance liquid chromatography*

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**Abstract**: The low turnover of caffeine *in vitro* by human liver microsomes makes the study of the metabolic pathways of this compound difficult. Analytical methods with high sensitivity and specificity are needed for the detection of its metabolic products. A method based on the on-line radiometric determination of [8C-$^3$H]caffeine and its principal metabolite (paraxanthine) in man has been developed using reversed-phase high-performance liquid chromatography. The method has been successfully employed in preliminary studies of the kinetics of this reaction.

**Keywords**: *On-line radio-HPLC; caffeine metabolism; enzyme kinetics; human liver microsomes; P450IA2.*

## Introduction

There is increasing interest in caffeine metabolism because of the possibility of using this compound as a model substrate in studies of the activity of some of the isoenzymes of cytochrome P450 involved in the toxicity of xenobiotics [1, 2]. Furthermore, there are a number of drug interactions with methylxanthines such as caffeine that, due to their widespread daily consumption, may have toxicological relevance for the general population [3]. The primary pathways of caffeine metabolism in rat and man are *N*-demethylations [4], which take place almost exclusively in the liver, catalysed by the cytochrome P450-dependent mixed function oxidase system. It has been suggested that the isoenzymes involved belong to the polycyclic aromatic hydrocarbon (PAH) inducible sub-family (P450IA), both in animals and in man [5, 6]. In recent studies with furafylline, a highly selective inhibitor of cytochrome P450IA2, we have established that the major route of caffeine metabolism in man, its *N*3-demethylation, is catalysed almost exclusively by this isoenzyme in the liver [7].

In previous studies of the kinetics of caffeine *N*-demethylation *in vitro* [8–10] high concentrations of substrate were used, due to the low turnover of caffeine. By using human microsomal fractions previously characterized as having high phenacetin *O*-deethylase (POD) activity (low $k_m$ component; a reaction catalysed by cytochrome P450IA2), together with radiolabelled substrate and analysis by radiometric-HPLC (with homogeneous on-line detection), sufficient increase in sensitivity was achieved to enable the accurate characterization of the kinetics of caffeine demethylation. This method can utilize lower concentrations of substrate than those used previously, and closer to those occurring *in vivo*.

## Materials and Methods

### *Chemicals*

[8C-$^3$H]Caffeine, with specific activity 22.2 Ci mmol$^{-1}$ (radiochemical purity 98%) was obtained from Amersham International (Bucks, UK). $NADP^+$, glucose 6-phosphate (G6P) and glucose 6-phosphate dehydrogenase (G6PDH), caffeine (CF), theophylline (TP), theobromine (TB), paraxanthine (PX), 7-methylxanthine (7X) and 1,7-dimethyluric acid (17U) were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). $MgSO_4$ was from Merck (Darmstadt, FRG). Ready Flow III (Beckman Instruments Inc., CA, USA) was

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used as liquid scintillant. Deionized water (Milli-Q water system, Millipore, Molsheim, France) and HPLC grade acetic acid (Scharlau, Ferosa, Barcelona, Spain), methanol and acetonitrile (Merck, Darmstadt, FRG) were used for the mobile phase.

*Apparatus*

HPLC analyses were performed using a modular liquid chromatographic system, consisting of models 112 and 110B solvent delivery modules, a model 165 variable wavelength detector and a model 171 radiometric detector from Beckman Instruments Inc. (CA, USA) together with an automatic injector (model ISS-100) from Perkin–Elmer Ltd (Beaconsfield, UK). Data acquisition and reduction were performed using a model LCI-100 integrator (Perkin–Elmer Ltd) and a PC-based computer system running M-171 laboratory software (Beckman Instruments Inc.).

*Column and mobile phase*

Separations were achieved using a C18 reversed-phase column (Ultrasphere ODS 5 μm; 0.46 × 25 cm, Beckman). The mobile phase comprised a mixture of acetic acid (0.05%)–methanol–acetonitrile (87:11:2%, v/v/v). Chromatography was performed isocratically at ambient temperature at a flow rate of 1 ml $min^{-1}$. The elution times of CF and its oxidative products were determined by monitoring UV absorption at 280 nm. This was also used to quantify the recovery of internal standard. Liquid scintillant was pumped continuously (flow rate 3 ml $min^{-1}$, i.e. a ratio of scintillant to eluent of 3:1) into the chromatographic eluent and the mixture led through the flow-cell of the radiometric detector, which was used to quantify recovery of caffeine and any metabolic products. Retention times for 7X, 1X, TB, PX, TP, 17U and CF under these conditions were 5.2, 7.6, 9.6, 15.4, 17.0, 18.6 and 33.5 min, respectively.

In vitro *assay of caffeine metabolism*

The metabolism of caffeine *in vitro* was determined by a modification of the methods of Bonati *et al.* [8] and Grant *et al.* [9]. Incubation mixtures contained, in a final volume of 0.5 ml, 31 mM KCl, sufficient unlabelled caffeine to give final concentrations of 0, 10, 25, 100, 250, 500, 1000 and 5000 μM), [8C-$^3$H]caffeine (2 μCi), an NADPH generating system comprising 2 mM G6P, 0.2 unit $ml^{-1}$ G6PDH, 0.2 mM $NADP^+$ and 1 mM $MgSO_4$. The volume of the samples was adjusted to 0.4 ml with 0.2 M $KH_2PO_4$/$K_2HPO_4$ buffer, pH 7.4. After preincubation for 5 min at 37°C, the reaction was started by the addition of 2 mg of microsomal protein (in 0.1 ml of 0.25 M potassium phosphate buffer, pH 7.25, containing 30% glycerol) to the samples. The samples were incubated in a shaking water bath (Kottermann 3047) at 37°C in an air atmosphere for 30 min, after which the reaction was terminated by the addition of 10 ml of a mixture of ethyl acetate–chloroform–isopropanol (45:45:10%, v/v/v), and internal standard (10 μg 7X in 100 μl phosphate buffer) followed immediately by vigorous mixing for 15 s using a vortex-mixer (Reax-2000, Heidolph, GDR, speed 8). Ammonium sulphate (0.5 g) was then added to the samples, followed by vortex mixing for a further 60 s, then centrifugation at 1500 ***g*** for 5 min to separate the aqueous and organic phases. The organic extracts were evaporated to dryness under a stream of nitrogen at 40°C (N-Evap 111, Organomation Assoc. Inc., MA, USA) and the residues redissolved in 100 μl of methanol. Reconstituted samples were injected (40 μl) onto the radio-HPLC system.

*Calibration procedure*

To check the linearity of the response of the UV detector, 40 μl of solutions of different concentrations of PX, TB, TP, 1X, 17U and CF, dissolved in 0.2 M phosphate buffer, pH 7.4, were injected on to the chromatographic system, using the same conditions as described above. The amounts injected corresponded to concentrations of 0, 4.0, 10, 25, 50 and 75 μg $ml^{-1}$ in the incubation samples. The standards were injected in triplicate. To calibrate the radiometric detector, samples containing serial dilutions of radiolabelled [8C-$^3$H] caffeine in buffer were prepared. Forty μl of each sample, corresponding to concentrations of 1.0, 2.5, 10, 20, 40 and 80 μg $ml^{-1}$ in the incubation samples, were injected onto the radio-HPLC system.

To check recovery through the method, triplicate sets of four concentrations (2.0, 6.0, 12.0 and 24.0 μg $ml^{-1}$ of each of the possible oxidative metabolites indicated under Chemicals) were incubated using the same conditions as described above, except that microsomal protein was not added until after termination

of the incubation. These samples were analysed using UV-detection.

Statistical analyses were performed using a commercial software package (Statgraphics 2.1, Statistical Graphics Corp., USA).

## Results

*Linearity of detector response and recovery of caffeine and its metabolites*

The response of the UV-detector was linear over the range of concentrations studied; TP, $r = 0.9989$; PX, $r = 0.9988$; TB, $r = 0.9994$; IX, $r = 0.9986$; 17U, $r = 0.9990$; and caffeine, $r = 0.9989$. The radiometric detector also gave a linear response, with [8C-$^3$H]caffeine ($r = 0.9992$). The limit of detection of [8C-$^3$H]caffeine was 0.1 ng (corresponding to a concentration of 0.5 ng ml$^{-1}$ in the incubation samples), with a signal to noise ratio of 3.5. With the conditions used for the assay, recovery of all compounds from the incubation mixture was in the range 85–100%.

*Characteristics of human liver samples*

Microsomal fractions from three different human livers (03007, 03008 and 03009) were characterized as previously described [7]. Activities for high affinity POD ($V_{max}$) for the samples studied are shown in Table 1. Two of the samples, 03007 and 03009, had relatively high activity whilst the third (03008) had much lower activity.

*Kinetics of caffeine metabolism*

Only PX, the principal metabolite of CF in man, produced by its *N*3-demethylation, could be detected (Fig. 1) following the incubation of [8C-$^3$H]caffeine with human liver microsomes, and only with two of the three samples studied. The rates of formation of the other oxidation products of caffeine were below 1 pmol mg$^{-1}$ min$^{-1}$, the limit of detection of the assay.

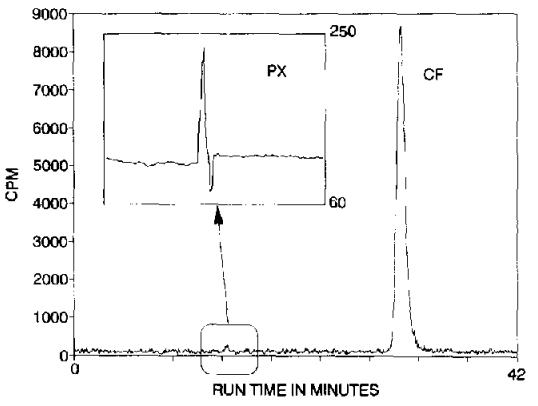


**Figure 1**
Radio-HPLC chromatogram of sample 03009 using 10 μM of caffeine. PX, paraxanthine; CF, caffeine.

The kinetics of PX formation were determined over a range of concentrations of caffeine (10–5000 μM), using the two samples of human liver with which this metabolite could be detected, by linear regression analysis of the two components observed in the Eadie–Hofstee plots (Fig. 2). The values of $K_m$ and $V_{max}$ thus obtained for the high affinity component and, tentatively ($n = 3$ points), for the low affinity component are shown in Table 1.

## Discussion

A radio-HPLC method has been developed for the assay of caffeine metabolism *in vitro*, which enables concentrations of the substrate lower than those used previously [9–11] to be studied. This has permitted more accurate determination of the kinetics of the high affinity component of the *N*3-demethylation of caffeine. The estimates obtained for $V_{max}$ and $k_m$ using samples 03007 and 03009 are in agreement with those reported by other groups [9, 11], using samples with high activities characteristic of P450IA2. PX formation could be detected only with two of the samples

**Table 1**
Results of microsomal characterization and caffeine kinetics of *N*3-demethylation pathway

| Human liver sample | Phenacetin *O*-deethylase (pmol min$^{-1}$ mg$^{-1}$) | Caffeine kinetics* High-affinity site ($K_m$) | High-affinity site ($V_{max}$) | Low-affinity site ($K_m$) | Low-affinity site ($V_{max}$) |
|---|---|---|---|---|---|
| 03007 | 135 | 0.9 | 145 | 28 | 3300 |
| 03008 | 4 | ND† | ND | ND | ND |
| 03009 | 90 | 1.0 | 120 | 59 | 4000 |

*Units: $K_m$, mM; $V_{max}$, pmol min$^{-1}$ mg$^{-1}$.
†ND, not detected.

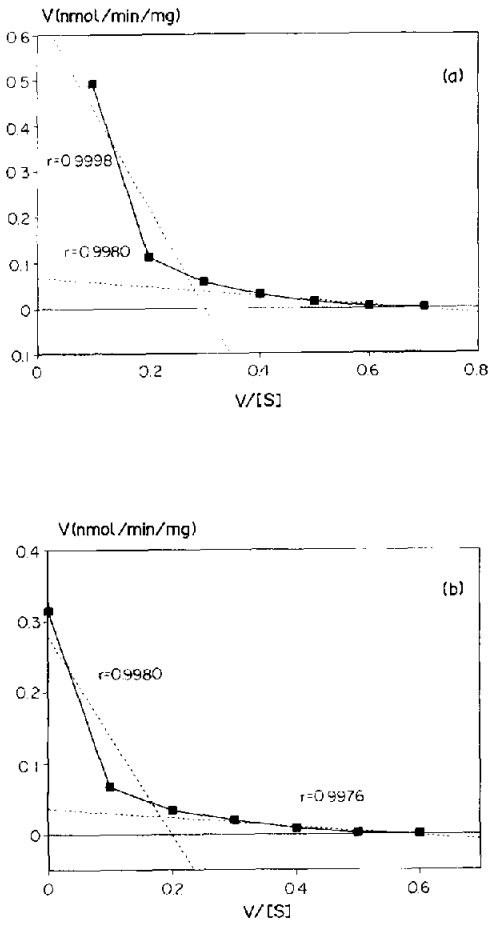


**Figure 2**
Caffeine kinetics. Eadie–Hofstee representation: (a) sample 03007 (b) sample 03009. Heavy solid lines represent the observed reaction velocities. Dashed lines represent the linear regression for the high- and low-affinity sites, respectively.

studied. These had high activity for phenacetin *O*-deethylase, whilst POD activity of the third sample (03008), with which PX formation could not be detected, was almost 30-fold lower than that of the other two samples. This is consistent with the involvement of P450IA2 in both reactions [7, 11].

Despite the inability to detect high affinity PX activity with sample 03008 using the radio-HPLC method, it was shown previously, using the HPLC method of Grant *et al.* [9] with UV-detection, that this sample did produce detectable amounts of PX at high concentrations of caffeine (1 mM). Failure to detect PX activity using the radio-HPLC method was a consequence of using a constant amount of radiolabelled caffeine in the incubations. As a result of this, the sensitivity of the assay decreases with increasing substrate concentration. The sensitivity of the assay could be improved by increasing the final specific activity of caffeine in the incubations, at all substrate concentrations.

The products of the other oxidation pathways of caffeine metabolism were not detected following incubation of caffeine with human liver microsomes ($n$ = 3) using radio-HPLC. However, such products could be detected when a high concentration of caffeine (1 mM) was employed [7]. This is in agreement with observations *in vivo* that *N*3-demethylation is the major route of metabolism of caffeine.

The *N*3-demethylation of caffeine has the potential of serving as a convenient means of assessing, both *in vivo* and *in vitro*, the activity of cytochrome P450IA2, an enzyme of considerable toxicological concern, as it is involved in the activation of a number of carcinogenic compounds to their genotoxic products [11]. As both the present study and previous work have demonstrated, there is considerable interindividual variation in caffeine *N*3-demethylase activity. Thus, to enable routine estimation of this activity, even in samples in which it is low, it is necessary to increase the sensitivity of the radio-HPLC assay. This will be achieved by increasing the specific activity of caffeine in the incubation, and by maintaining the specific activity with increasing concentrations of substrate.

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