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Subnanomolar quantification of caffeine's in vitro metabolites by stable isotope dilution gas chromatography–mass spectrometry

Kelly A. Regal, William N. Howald, Raimund M. Peter, Carlos A. Gartner, Kent L. Kunze, Sidney D. Nelson*

Department of Medicinal Chemistry, School of Pharmacy, Box 357610, University of Washington, Seattle, WA 98195, USA

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Abstract

A method for the quantification of subnanomolar levels of in vitro metabolites of caffeine by an isotope dilution gas chromatographic–mass spectrometric (GC–MS) assay has been developed and applied. Trideuteromethylated analogs of each primary metabolite were synthesized and added after incubations of caffeine with human liver microsomes high in cytochrome P4501A2. HPLC separation of the metabolites prior to GC–MS quantification allowed the isolation of theobromine and paraxanthine which coeluted by GC and enabled quantification over a larger dynamic range. Quantitative analysis was performed on the *n*-propylated derivatives by selected-ion monitoring of either the $M^{+\cdot}$ ions for the dimethylxanthines or $[M-C_3H_6]^{+\cdot}$ ions for 1,3,7-trimethyluric acid. For the least abundant metabolite (1,3,7-trimethyluric acid), the detection level on column was 200 pg. Replicate analyses exhibited intra- and inter-day variability of 4.2 and 7.9%, respectively. This assay has been successfully used in the quantification of caffeine's primary metabolites in more than 180 incubations, at varying substrate concentrations and with multiple enzyme sources. © 1998 Elsevier Science B.V.

Keywords: Stable isotope dilution GC–SIM-MS; Caffeine; Paraxanthine; Theobromine; Theophylline; 1,3,7-Trimethyluric acid

1. Introduction

Caffeine is metabolized to four primary metabolites by cytochrome P4501A2 (CYP1A2) in vitro (Fig. 1) [1]. In humans, the major metabolite of caffeine is paraxanthine (PX), formed by *N*-demethylation of the N-3 position [2]. This is also observed with in vitro experiments utilizing human CYP1A2 (hCYP1A2). CYP1A2 is believed to be completely responsible for PX formation in vivo and to partially contribute to the formation of theobromine (TB), theophylline (TP), and 1,3,7-trimethyluric acid (TMU). The ratio of metabolite formation by hCYP1A2 is approximately 80:11:4:1 PX:TB:TP:TMU [2,3].

Previous studies indicate that substitution of deuterium for metabolically susceptible hydrogen(s) can decrease metabolism at that position with no concomitant change in the overall extent of metabolism [4–6]. Thus, as the rate of formation of one metabolite is decreased by the presence of a carbon–deuterium bond, there is a commensurate increased rate of formation of one or all of the alternate metabolites. Our goal was to develop an assay which

*Corresponding author

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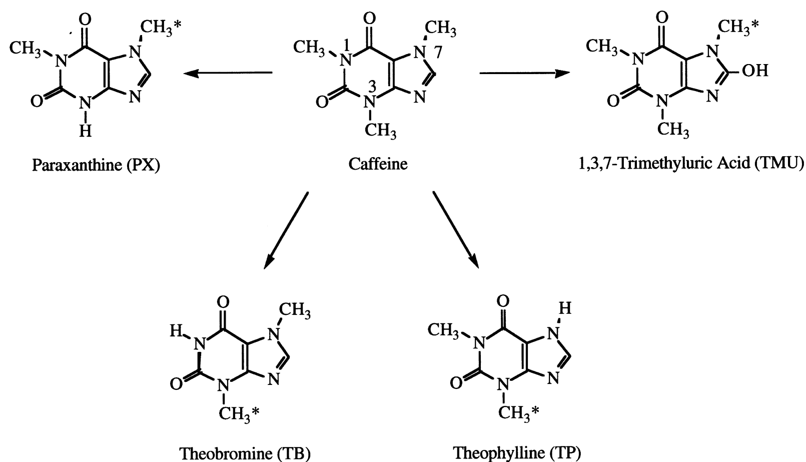

Fig. 1. Primary metabolism of caffeine; * indicates sight of stable label on corresponding I.S.

would enable investigation of this phenomena when the N-3 methyl group of caffeine is replaced with a trideuteromethyl (deuteromethyl) group. Thus an assay capable of detecting small changes in the levels of metabolites and the overall turnover as well as changes in the expected ratio of formation of the four primary metabolites was required.

Generally, quantification of caffeine metabolites utilizes HPLC separation with UV detection [7–13]. Various problems have been encountered with this approach, including inefficient extraction of metabolites, overlap of contaminant and metabolite peaks, lack of separation between certain pairs of primary metabolites and inadequate sensitivity. Protocols for quantification by GC–MS have been published [14–17], but were found to be inadequate for our purposes. We were unable to analyze underivatized metabolites following the methods of Kumazawa et al. [14]. The method of Horning et al. [15] did not quantitate TMU, does not resolve the dimethylxanthines and employed a single internal standard which does not display the same extractability as the primary metabolites. Benchekroun et al. [16,17] were also unable to separate TB from PX. Since the solubilities and pK_a values of these metabolites vary significantly [18], the application of stable-labelled internal standards (I.S.s) for each primary metabolite, in combination with quantification by GC–MS was required [19]. Baseline separation of all the primary metabolites was also essential for quantification over a large dynamic range.

Thus, a stable isotope dilution method involving the RP-HPLC separation and GC–MS quantification of the primary metabolites of caffeine, from in vitro systems, was developed. We believe this to be the first GC–MS method capable of quantitating caffeine's primary metabolites at the ratio they are formed in vitro, by hCYP1A2 or human liver microsomes.

2. Experimental

2.1. Chemicals and materials

Caffeine, TB, TP and TMU were purchased from Sigma (St. Louis, MO, USA) and further purified by preparative HPLC to remove trace amounts of contaminating xanthines. 1-Methylxanthine, 7-methylxanthine and 8-chloro-theophylline were obtained from Sigma. PX was obtained from Fluka (Buchs, Switzerland). Dimethylformamide (DMF) was obtained from Aldrich (Milwaukee, WI, USA), stirred with potassium hydroxide (KOH), vacuum distilled from calcium oxide [20] and stored over molecular sieves (4 Å, Aldrich). Reagent-grade potassium carbonate (K_2CO_3; Aldrich) was dried for 16 h in a vacuum oven (>100°C). 1-Iodopropane (Aldrich) was analyzed by GC for contaminants. If contaminants were greater than 0.5% of the total peak area, it was further purified by distillation. Deuteromethyliodide (C^2H_3I or CD_3I; $\%d_3=99\%$;

Aldrich) was stored at −20°C to avoid evaporation. HPLC solvents were reagent grade. All other chemicals and solvents were analytical grade.

2.2. Instrumentation

Reaction monitoring and RP preparative purifications were performed on an HP1090 Series IIL liquid chromatograph equipped with a DR5 ternary solvent delivery system, a temperature-controlled autoinjector and column compartment, a built-in Diode Array Detector Series II and a DOS system control unit (Hewlett-Packard, Wilmington, DE, USA). Normal-phase chromatography was performed on an LKB Model 2152-2SD dual-pump instrument equipped with an LKB Model 2151 variable-wavelength detector. NMR measurements were performed on a Varian VXR-300 FT-NMR (Varian Instruments, Palo Alto, CA, USA). Deuterium incorporation measurements were carried out on a Micromass 7070H GC–MS (Micromass, Cheshire, UK).

GC–MS analyses were performed on a Micromass Trio 2000 quadrupole mass spectrometer, fitted with a Hewlett-Packard 5890 Series II gas chromatograph that was equipped with a capillary splitless injector (Hewlett-Packard, Palo Alto, CA, USA) and a Hewlett-Packard 7673A autosampler. A BPX5 fused-silica capillary GC column (30 m×0.32 mm I.D., 0.25 μm film thickness; 5% phenyl polysilphenylene–siloxane; SGE, Austin, TX, USA) was used and operated with helium (head pressure, 0.69 bar) as carrier gas. Samples were injected in the splitless mode (injector temperature, 250°C) using a purge delay of 1 min on injection and a 3 ml/min septum purge. The GC was programmed at 150°C, for 1 min, and then ramped at 20°C/min to 210°C and held for 1 min and, finally, ramped at 5°C/min to 250°C where the temperature was maintained 5 min.

The mass spectrometer was operated in the positive ion mode with a filament emission current of 200 μA and an electron energy of 70 eV. The ion source temperature was 200°C and the GC–MS interface temperature was maintained at 250°C. Ion windows (±0.25 Da) for selected-ion monitoring (SIM), data acquisition and daily tuning of the quadropole were accomplished using 486DX50x2 PC and Micromass MassLynx software. Ions monitored for the derivatized dimethylxanthines correspond to the H_3 and $^2H_3$ molecular ions (d_0 and d_3; *m/z* 222.1 and 225.1). In the case of the derivatized TMUs (d_0 and d_3), the respective base peaks at *m/z* 210.1 and 213.1 were monitored. The dwell times were set at 50 ms per ion.

2.3. Synthesis

Synthesis of I.S.s for the *N*-demethylated metabolites of caffeine (7-CD_3-PX, 3-CD_3-TP and 3-CD_3-TB) followed the methods of Berlioz et al. [21] with some modifications. Briefly, deuteromethylation was initiated with oven-dried K_2CO_3, CD_3I and dry DMF as solvent. Additional aliquots of base and alkylating agent were added to force the reaction to completion. The reaction was monitored by RP-HPLC (Alltech Econosil C18 (Alltech, San Jose, CA, USA); 250×4.6 mm; 5 μm; 10:90% CH_3CN–H_2O over 10 min, held for 2 min, 90:10% CH_3CN–H_2O over 3 min, held for 5 min; λ=270 nm). Upon completion, the DMF was evaporated and the compounds were purified to remove contaminating dimethylxanthines. Preparative HPLC was found to be the most efficient method of purification. Each deuteromethylated xanthine exhibited identical HPLC retention times and UV spectra to the corresponding commercially available compounds. NMR analysis gave chemical shifts which match those reported by Zbaida et al. [22]. In all cases, residual protons on the deuteromethylated carbon could not be observed by NMR. All compounds were stored at 4°C, in a desiccated flask. All reactions and analyses were performed at ambient temperature unless otherwise noted.

2.4. 3-C^2H_3-Theobromine (3-CD_3-TB)

7-Methylxanthine (100 mg; 0.60 mmol) was mixed with 1.5 molar equiv. of dry K_2CO_3 and 1.1 molar equiv. of CD_3I in 10 ml of dry DMF, and the flask was flushed with argon. After 14 h at room temperature, the reaction was essentially complete and the DMF was evaporated under vacuum. The residue was redissolved in 2.5 ml of slightly basic H_2O and injected onto a preparative RP-HPLC column (Beckman Ultrasphere ODS column (Alltech); 250×10 mm; 5 μm) in 0.05% CH_3COOH–8%

MeOH–H_2O, at a flow-rate of 5 ml/min. The monitored wavelength was 274 nm. The product eluted at approximately 18 min, with residual starting material appearing at 5 min and TP–PX at 31 min. The $^2H_3$ compound was 99.2% of the $^2H_3$ isotopomer, 0.5% $^2H_2$, 0% $^2H_1$ and 0.3% $^2H_0$. The overall yield was 12%.

2.5. 7-C^2H_3-Paraxanthine (7-CD_3-PX)

A solution of 10 ml dry DMF, 1-methylxanthine (250 mg; 1.5 mmol) and one molar equiv. of dry K_2CO_3 was flushed with argon, followed by addition of 1.1 molar equiv. of CD_3I. After stirring at room temperature, for 6.5 h, the reaction appeared to be complete and the DMF was evaporated under vacuum. After a quick screen by RP-HPLC indicated there was no TB present in the mixture, the product (in 10 ml of $CHCl_3$) was injected onto a preparative HPLC column (Whatman Partisil 10 (Fisher Scientific, Santa Clara, CA, USA); 500×9.4 mm; 5 μm) and eluted isocratically in a mobile phase of 70% isopropanol (IPA)–hexane at a flow-rate of 2 ml/min. Products were detected at 272 nm. The retention time of 7-CD_3-PX was approximately 40 min, with the side products eluting both before (TP, 28 min) and after (caffeine, 60 min). The $^2H_3$ compound was 99.0% of the $^2H_3$ isotopomer, 0.5% $^2H_2$, 0.2% $^2H_1$ and 0.3% $^2H_0$. The overall yield was 12%.

2.6. 3-C^2H_3-Theophylline (3-CD_3-TP)

1-Methylxanthine (60 mg; 0.36 mmol) was dissolved in 10 ml of dry DMF and 1.5 molar equiv. of dry K_2CO_3 were added to the stirring mixture. After flushing with argon, 1.1 molar equiv. of chloromethyl pivalate were added. Monitoring of the reaction by RP-HPLC indicated that protection at the N-7 position was favored over the N-3 position [18] with the presence of some doubly protected (N-7 and N-3) 1-methylxanthine. After 9 h, less than 10% starting material remained and the deuteromethylation was begun by adding one molar equiv. of CD_3I. When less than 6% of the mono-protected 1-methylxanthine remained, the DMF was evaporated. Deprotection was then carried out by dissolving the residue in 10 ml of 2 *M* NaOH, followed by lyophilization. The reaction mixture was taken up in a few ml of 10% MeOH–$CHCl_3$, loaded onto a silica column (60×19 mm; Kieselgel 60, 70–230 mesh, E. Merck, Darmstadt, Germany) and sequentially washed with 200-ml aliquots of 20 and 50% MeOH–$CHCl_3$. The eluent was monitored by TLC (commercially precoated silica gel 60 F_{254} with fluorescent indicator activated at 254 nm; 55×10 mm, with the starting position 5 mm off the bottom; 0.25 mm thickness (Aldrich); R_F (TP)=0.43, R_F (PX)=0.36, R_F (caffeine)=0.53 in 2 ml 10% MeOH–$CHCl_3$ and a 60×25 mm I.D. chamber (Alldrich)). After combining the relatively caffeine-free fractions and evaporating the solvent, the product (in 3 ml of $CHCl_3$) was injected onto the preparative HPLC column under conditions identical to those used for 7-CD_3-PX. The $^2H_3$ compound was 97.7% of the $^2H_3$ isotopomer, 1.8% $^2H_2$, 0.1% $^2H_1$ and 0.6% $^2H_0$. The overall yield was 21%.

2.7. 7-C^2H_3-Trimethyluric acid (7-CD_3-TMU)

An alternate synthetic strategy was used [23] and modified as follows. Starting with the same deuteromethylation procedure described for 7-CD_3-PX and 3-CD_3-TB, 8-chlorotheophylline (250 mg; 1.17 mmol) was deuteromethylated, for 14 h, in the presence of 1.5 molar equiv. of dry K_2CO_3, 1.1 molar equiv. of CD_3I and 10 ml of dry DMF. The reaction was monitored by TLC (same general conditions as 3-CD_3-TP; R_F (8-chlorotheophylline)= 0.68, R_F (8-chlorocaffeine)=0.92 in 2 ml 10% EtOH–$CHCl_3$). The DMF was evaporated with no purification of the intermediate. Following resuspension in EtOH, 2.5 molar equiv. of KOH in EtOH were added and the temperature brought up to reflux (100–105°C). After 3 h, the reaction was greater than 90% complete as determined by RP-HPLC and TLC (R_F (8-ethoxycaffeine)=0.82 in 10% EtOH–$CHCl_3$). Evaporation of the EtOH was followed by resuspension in an excess of 16% (v/v) HCl. After 10 min at 100°C, the reaction was complete (R_F (TMU)=0.43 in 10% EtOH–$CHCl_3$). The product was purified by preparative RP-HPLC (Beckman Ultrasphere ODS column (Alltech); 250×10 mm; 5 μm). The method employed 0.05% CH_3COOH–12% MeOH–H_2O as the mobile phase, delivered at a

flow-rate of 5 ml/min while monitoring 290 nm. Under these conditions, the retention time of 7-CD_3-TMU was approximately 25 min. The $^2H_3$ compound was 99.7% of the $^2H_3$ isotopomer, −0.5% $^2H_2$, 0.2% $^2H_1$ and 0.3% $^2H_0$. The overall yield was 59%.

2.8. Caffeine purification

Commercially available caffeine was dissolved in $CHCl_3$ and injected onto a preparative HPLC column (Whatman Partisil 10 (Fisher Scientific); 500×9.4 mm; 5 μm) which had previously been equilibrated in 5% IPA–$CHCl_3$. The flow-rate was 5 ml/min and the monitored wavelength was 270 nm. Caffeine eluted isocratically with a retention time of 24 min.

2.9. Caffeine assay: incubations

Human liver microsomes were prepared as previously described [24]. Cytochrome P450 (P450) specific content was determined by the method of Omura and Sato [25] and protein was determined by the BCA method (Pierce Chemical Co., Rockford, IL, USA; [26]). Incubation conditions were similar to those reported by Gu et al. [1], with some modifications. Our conditions were shown to be linear over the time and protein content used. Briefly, the incubation mixture of 0.45 ml contained the following: 5 m*M* caffeine, 20 μl of 1 *M* phosphate buffer (pH 7.4), 20 μl of 5.75% KCl, 20 μl of 4.75% $MgCl_2$ and 1 nmol of human liver microsomes, shown to be high in CYP1A2 content (HL103). The mixture was preincubated for 3 min at 37°C and the reaction started with the addition of 50 μl of an NADPH regeneration system (45 μl 46.5 mg/ml glucose 6-phosphate, 2 μl 700 units/ml of glucose 6-phosphate dehydrogenase and 3 μl 86.5 mg/ml $NADP^+$). After incubating at 37°C for 60 min, the reaction was stopped by the addition of 100 μl of 1 *M* HCl, followed by addition of 50 μl of the I.S. mixture (80:11:4:1 7-CD_3-PXL:3-CD_3-TB:3-CD_3-TP:7-CD_3-TMU), 100 μl of 1 *M* ammonium sulfate and 5 ml of 4:1 CH_2Cl_2–IPA. After shaking for 30 min, the samples were centrifuged at 1500 *g* for 10 min, the organic layer separated and evaporated. This extraction procedure was repeated once and the solvent evaporated with N_2. Dry samples were stored at −20°C until further analysis.

2.10. HPLC

The samples were resuspended in 200 μl of 5% CH_3CN–tetrahydrofuran (THF) (3:1) (solvent A), 95% 8.3 m*M* ammonium acetate, pH 4 (solvent B) and 175 μl of the sample were injected onto the HPLC column to separate the metabolites (Zorbax RX-C8 (MAC-MOD Analytical, Inc., Chadds Ford, PA, USA); 75×4.6 mm; 3.5 μm). The metabolites eluted isocratically in a mixture of 5% (A), 95% (B), at a flow-rate of 0.7 ml/min with the column temperature set at 40°C. The overall run time was 15 min. Fractions containing PX, TB and the combined TP–TMU metabolites were collected in concentration tubes (Laboratory Research, Lomita, CA, USA), dried by lyophilization and stored at −20°C until further analysis.

2.11. Derivatization

Approximately 5 mg of dry K_2CO_3 were added to the tubes containing the TP and TMU samples dissolved in 100 μl of dry DMF. After heating to 65°C for 30 min, 100 μl of iodopropane were added, the samples vortexed and held at this temperature. RP-HPLC analysis showed the derivatization to be complete after 2 h. Following the addition of a few mg of sodium bisulfite, the DMF was evaporated under vacuum. Heating the samples while under vacuum was avoided in order to minimize loss of the metabolites by sublimation. The samples were resuspended in 60 μl of CH_3CN and were transferred to 1.5-ml vials with 200-μl conical-bottom inserts for GC–MS analysis.

TB and PX samples were dissolved in 100 μl of dry DMF. After the addition of 10 μl of 1 *M* KOH in MeOH and heating for 30 min at 30°C, 100 μl of iodopropane were added and the reaction allowed to proceed for 24 h at this temperature. Following addition of a few mg of sodium bisulfite, the DMF was evaporated (no heat as mentioned above). The samples were then resuspended in 100 μl of CH_3CN and transferred into 1.5-ml vials with 200-μl flat-bottom inserts for GC–MS analysis.

2.12. Standard curves

In order to demonstrate a linear response by GC–MS for each of the four primary metabolites over the necessary quantification range, known ratios of I.S. and metabolite (1:15, 1:3, 1:1, and 3:1, centered around the expected amount of the respective metabolite) were mixed, derivatized as described above and analyzed by GC–MS. A representative plot of known vs. observed peak area ratios of metabolite and the corresponding I.S., over a 50-fold range, is shown in Fig. 5. Also, the inset shows the on-column detection of TMU, at a 100:1 signal-to-noise ratio.

In order to determine the intra- and inter-day variability, equimolar amounts of the I.S. mixture (80:11:4:1 7-CD_3-PX:3-CD_3-TB:3-CD_3-TP:7-CD_3-TMU) and a corresponding mixture of the commercially available compounds were mixed. The amounts added were representative of expected metabolite formation at the lowest caffeine concentration to be used in our assay (100 μM). This mixture of d_0 and d_3 metabolites was added to human liver microsomes and the volume adjusted to 0.5 ml with H_2O. Following acidification and extraction, the peaks were purified by HPLC, and then derivatized and analyzed by GC–MS. Reported intra- and inter-day variabilities (Table 2) were the result of 10 replicate injections of the same sample and three replicate sample preparations, respectively.

3. Results and discussion

The goal of these efforts was to develop an assay with subnanomolar detection limits for the four primary metabolites of caffeine. The variation in solubility and pK_a values for these metabolites [18] necessitated the use of the corresponding stable isotope analogs for each of the analytes [19]. The use of stable isotope-labelled metabolites also eliminated the need for the determination of recovery for each of the metabolites. In each case, a deuteromethyl group was substituted for a methyl group, creating an analog 3 Da higher in mass than the metabolite. This facilitated quantification by providing complete separation between the isotopic envelopes of the analytes and the ions of the I.S.s used in the SIM-MS method.

Synthesis of 7-CD_3-PX and 3-CD_3-TB was straightforward due to the differential reactivity of the nitrogens in the xanthine ring system [18]. The acidic character of the N-7 position relative to N-3 required the protection of this position before deuteromethylation could be carried out in the synthesis of 3-CD_3-TP. The method of Balaban [23], starting with 8-chlorotheophylline, was utilized to synthesize 7-CD_3-TMU.

Purification of the stable-labelled metabolites by column chromatography, crystallization or preparative TLC did not remove trace levels of contaminating xanthines. Also, there was a significant reduction in yield when these methods were employed. Preparative HPLC completely removed trace levels of the contaminants with almost quantitative recovery. Normal-phase chromatography readily separated the $CHCl_3$-soluble PX, TP and caffeine from contaminating xanthines while RP-HPLC was required for the purification of TB and TMU.

Conditions were developed, using *n*-iodopropane in dry DMF, to prepare the stable *N*-alkylated derivatives [27]. Initially, these reactions used dry K_2CO_3 as the base. Derivatization of TB and PX was incomplete, even over extended periods of time, presumably due to differences in pK_a values between the metabolites [18]. However, using methanolic KOH as an alternate base resulted in the smooth conversion of PX and TB to a single alkylated product within an 8- and 12-h period, respectively. Propylation of TMU and TP was complete after 2 h at 65°C. While TP was derivatized to one product, TMU yielded two chromatographically distinct products which possessed different UV, HPLC, GC and MS properties. These are believed to correspond to the *N*- and *O*-alkylated derivatives (Fig. 2). Following derivatization, DMF was evaporated under vacuum.

Successful separation and MS quantification of the four primary metabolites, in the anticipated ratio of formation resulting from in vitro exposure of caffeine to hCYP1A2, is complicated by several factors. Being geometrical isomers, PX, TB and TP or their derivatives are isobaric sets with respect to diagnostic ions suited to their MS quantification [14,16]. Since the PX concentration is expected to be 20 and 80 times that of TP and TMU, respectively, any on-line GC or LC method employed must provide baseline separation of these analytes. The GC sepa-

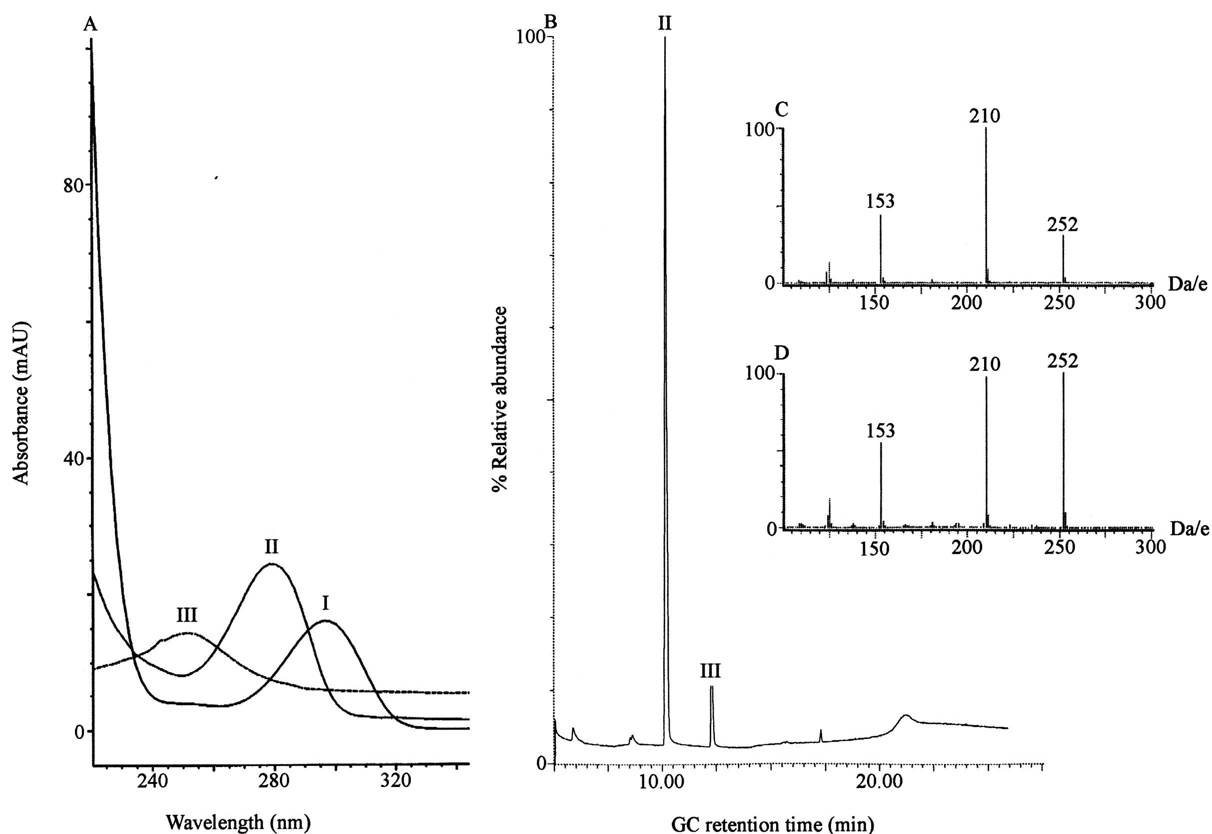

Fig. 2. Spectra of the two derivatization products of TMU. (A) UV spectra (in the order of elution by RP-HPLC) of (I) underivatized TMU, (II) '*O*-alkylated' TMU and (III) '*N*-alkylated' TMU. (B) Total ion current chromatogram of derivatized TMU by GC–MS. (C) Mass spectrum of (II). (D) Mass spectrum of (III). GC conditions similar to those in Table 1. Electron impact (EI)-MS conditions: electron energy, 70 eV; source temperature, 200°C.

ration of the dimethylxanthines achieved during preliminary work was limited to TP from unresolved TB and PX (*n*-propyl derivatives) on J&W DB-17 and DB-5 columns. Separation on the latter was observed only when the column was directly interfaced with the MS, i.e. a vacuum is applied to the column's terminus. The use of larger alkyl derivatives marginally enhanced separation. Attempts were made to develop an LC–MS assay utilizing either APCI (atmospheric pressure chemical ionization) or ESI (electrospray ionization) after RP-HPLC separation. In addition to the inherent MS difficulties described above, the level of detection for the TMU metabolite was found to be inadequate for these studies.

As a result, a two-step approach was developed which involved separation of the four metabolites into three fractions by RP-HPLC (1-TB, 2-PX, 3-TP–TMU; see Fig. 3), followed by GC–MS quantification of each component. This method allowed batching of samples by fraction (analyte(s)) and optimization of injected sample concentrations and MS detector gain for each. HPLC separation of PX from TP–TMU was necessitated by dynamic range considerations. Furthermore, with the removal of stringent requirements for GC separation, a general-use bonded-phase fused-silica capillary column could be used for analysis. Table 1 reports the relative GC retention index (in methylene unit (MU) values) for each metabolite relative to a series of *n*-alkane standards.

Since TMU was poorly extracted relative to the

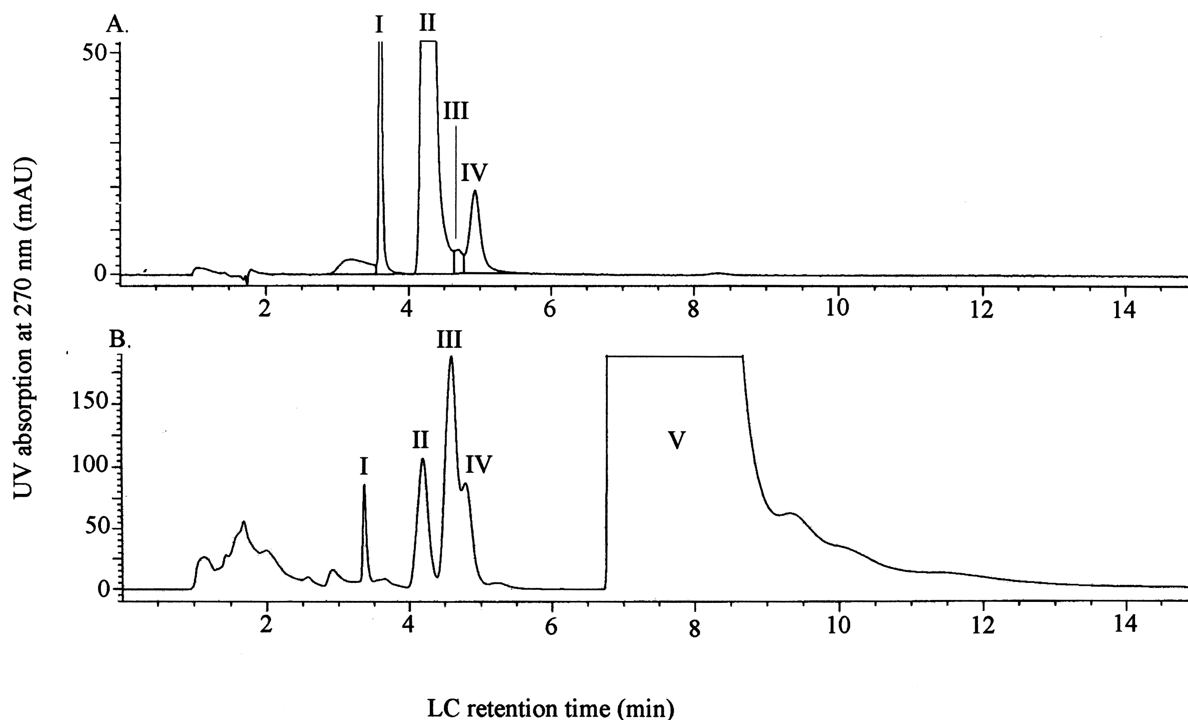

Fig. 3. HPLC chromatograms of (A) I.S.s at the expected ratio of formation by hCYP1A2 (80:11:4:1 7-CD_3-PX:3-CD_3-TB:3-CD_3-TP:7-CD_3-TMU) and (B) an extract from an incubation with human liver microsomes high in CYP1A2. (I) TB, (II) PX, (III) TMU, (IV) TP, (V) caffeine; 175 or 200 μl were injected onto a Zorbax RX-C8 column (75×4.6 mm; 3.5 μm) equilibrated in 5% CH_3CN–THF (3:1)–95% 8.3 m*M* ammonium acetate, pH 4. Column temperature, 40°C; flow-rate, 0.7 ml/min.

dimethylxanthines, and was expected to be formed to the least extent, extraction conditions were set to maximize TMU recovery. The use of 4:1 CH_2Cl_2–IPA was found to recover at least 75% of the TMU present. More polar solvent combinations were not selected in order to avoid the extraction of polar contaminants from the microsomes.

The alkylated derivatives of PX, TB, TP and TMU exhibited good chromatographic properties, i.e. having a peak width of less than 6 s at half height and

Table 1
GC retention indices and ions monitored for the in vitro metabolites of caffeine

Compound (*n*-propyl derivative)	Retention index (MU value)[a]	Ion monitored	
		m/z	Identity
PX	19.99	222.1	$M^{+}\cdot$
TB	19.96	222.1	$M^{+}\cdot$
TP	19.97	222.1	$M^{+}\cdot$
TMU	21.47,22.88[b]	210.1	$[M-CH_2CH_2CH_2]^{+}\cdot$

Samples and standards were analyzed on a 30-m DB5 (0.25 μm; J&W Scientific) column. The GC program started at 150°C, for 1 min, and then ramped up to 210°C at 10°C/min, held for another minute, ramped up to 250°C at 5°C/min and held for a further 5 min. Each sample was injected at least 5 times. Actual GC retention time: C_{18} 7.34±0.01 min; C_{20} 9.39±0.06 min; C_{22} 12.71±0.06 min; C_{24} 15.65±0.05 min. C.V.: PX, 0.2%; TB, 0.1%; TP, 0.1%; TMU, 0.3%.
[a]Methylene unit value as outlined in Section 2.
[b]Geometric isomers.

no observed interferences (Fig. 4). Derivatized TB, TP and PX were quantitated by monitoring the molecular ions of the metabolite and the I.S. (*m/z* 222.1 and 225.1). For derivatized TMU and its deuterated analog, 7-CD_3-TMU, the ion corresponding to $[M-C_3H_6]^{+\bullet}$ (*m/z* 210.1 and 213.1; Fig. 2B,C) were used for quantification.

Standard curves were generated from observed peak area ratios of the primary metabolites to their corresponding I.S. and were plotted against the known ratios of metabolite and I.S. The standard curves were found to be linear with correlation coefficients being greater than 0.99 in all cases over a 50-fold concentration range, centered around the amount of expected metabolite formation for each metabolite. A representative calibration curve is shown in Fig. 5. The on-column detection of 200 pg of TMU (signal-to-noise ratio of 100:1) is also shown. The actual quantification limits for the primary metabolites can be found in the footnotes of Fig. 5.

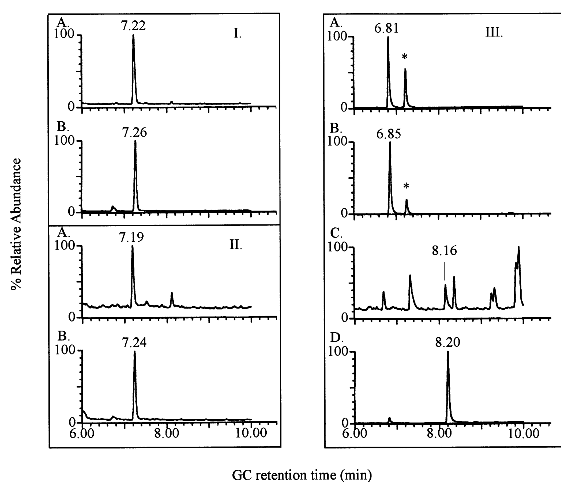

Fig. 4. Selected-ion current chromatograms of derivatized metabolites and their corresponding I.S. Three separate injections (1 μl each): (I) (A) 7-CD_3-PX (*m/z* 225.1) at 8.92 ng/μl, (B) PX (*m/z* 222.1) at 15.8 ng/μl; (II) (A) 3-CD_3-TB (*m/z* 225.1) at 1.23 ng/μl, (B) TB (*m/z* 222.1) at 3.61 ng/μl; (III) (A) 3-CD_3-TP (*m/z* 225.1) at 0.75 ng/μl, (B) TP (*m/z* 222.1) at 6.42 ng/μl, (C) 7-CD_3-TMU (*m/z* 213.1) at 0.22 ng/μl, (D) TMU (*m/z* 210.1) at 16.8 ng/μl. Labelled peaks are the peaks of interest. Samples were analyzed on a 30-m DB-17 (0.25 μm; J&W Scientific) column. The GC program started at 150°C, for 1 min, and then ramped up to 210°C at 30°C/min, held for another min, ramped up to 250°C at 5°C/min and held for a further 5 min. EI-SIM-MS conditions are the same as those reported in Table 2. *Residual 7-CD_3-PX and PX, respectively.

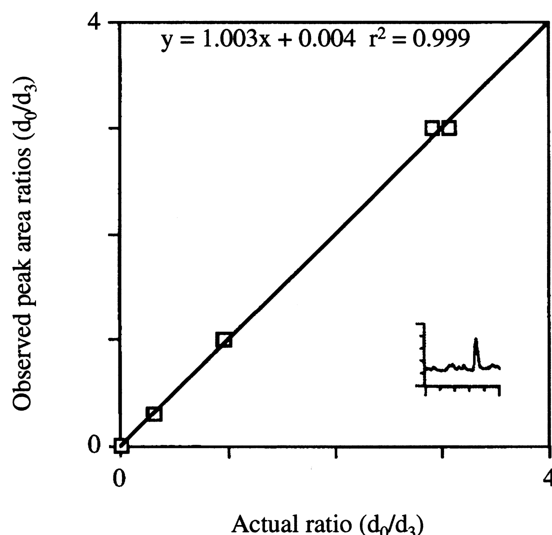

Fig. 5. Representative calibration curve for 7-CD_3-PX and PX (duplicate determinations). Peak area ratios were obtained by monitoring either the $M^{+\bullet}$ ions for *n*-propylated PX, TB and TP (*m/z* 222.1 and 225.1) or $[M-42]^{+}$. ions for *n*-propylated TMU (*m/z* 210.1 and 213.1). Inset: signal for 200 pg of *n*-propylated TMU (*S/N*, 100:1). Quantification limits: PX, 3.5 pg; TB, 4.8 pg; TP, 1.6 pg; TMU, <200 pg.

Data are given on the precision and accuracy of the assay in Table 2. Intra-day determinations were made by the analysis of two sample aliquots consisting of PX, TB, TP and TMU, mixed in the ratio of 80:11:4:1, with equivalent amounts of their deuterated I.S. The concentration of the metabolites in these samples were at levels expected to be representative of the lowest level of caffeine turnover in future sub-K_m incubations, assuming a V_{max} of 1 nmol min^{-1} nmol $P450^{-1}$ [1] and a K_m of 0.5 m*M*. Each sample was injected 10 times. Inter-day precision was obtained by quantitating metabolites from an incubation and the standards mixture, on three different days. The intra- and inter-day mean coefficients of variation never exceeded 8%.

Inter- and intra-day variability analysis by Benchekroun et al. [16,17] had similar results, with their largest variability seen when PX was present at 10 times the concentration of TB. Presumably this was due to coelution of PX and TB under their conditions, necessitating additional calculations. When

Table 2
Intra- and Inter-day coefficients of variation

Metabolite	Expected amount (ng)[a]	Intra-day variation (n=10)	Inter-day variation (n=3)
PX	450	452±2.2 (0.4%)	448.5±3.7 (0.8%)
TB	60	58.4±0.9 (3.8%)	60.3±3.3 (5.4%)
TP	23	25.6±1.1 (4.2%)	25.3±2.0 (7.9%)
TMU	6.3	7.0±0.3 (1.9%)	6.4±0.2 (3.1%)

GC conditions same as Table 1. EI-SIM-MS conditions: electron energy at 70 eV; source temperature at 200°C; $M^{+}\cdot$ (m/z 222.1) monitored for PX, TB, TP; $[M-C_3H_6]^{+}\cdot$(m/z 222.1) monitored for TMU; dwell, 50 ms per ion. Numbers in parentheses represent C.V.
[a]Expected to be representative of substrate turnover with hCYP1A2 at 100 μ*M* caffeine.

rat CYP1A2 is the enzyme source this is not a significant problem, since the formation ratio of PX–TB in such an in vitro system is 1:1 [28]. However, hCYP1A2 is known to form these two dimethylxanthines in an 8:1 ratio [2,3], and the goal of this work was to be able to detect small variations in this ratio. Variability in metabolite quantification by HPLC methods ranged from 5 to 30% [10–12]. Error analysis in the GC–MS methods of Kumazawa et al. [14] or Horning et al. [15] was not mentioned.

Incubations were performed with human liver microsomes, I.S. then added at the expected levels of caffeine turnover (1 nmol min^{-1} nmol $P450^{-1}$) and the metabolites separated by RP-HPLC, derivatized and quantitated by GC–MS. Fig. 3 shows the HPLC traces of both the I.S. mixture and a representative trace from an incubation. The ratio of metabolites is slightly different from those observed in the presence of hCYP1A2 alone, since several P450s are present. The most obvious difference is a large TMU peak. Fig. 4 shows representative GC–MS traces for each metabolite and their corresponding deuteromethylated I.S.

Upon repetitive injection (20–30 samples), a buildup of salt on the GC injection liner was observed, leading to a loss in sensitivity, especially for TMU. It is now believed that this salt is potassium iodide, a side product of the derivatization. As this salt decomposes, the derivatized metabolites also decompose if stored for any length of time. Decomposition is accompanied by the residue or solution turning yellow and then brown upon exposure to light, moisture and air [29], and this color change has been seen with some of the samples. These problems are avoided by adding a few mg of sodium bisulfite to the samples before evaporating the DMF. Presumably, the bisulfite prevents the oxidation of the potassium iodide to iodine and iodate, which can induce decomposition of the metabolites.

4. Conclusions

A stable isotope dilution GC–SIM-MS method, following metabolite separation by RP-HPLC, has been developed and proven to be reliable, accurate and precise in the quantification of the in vitro metabolites of caffeine. The specificity and sensitivity of GC–MS assays were enhanced by the use of $[^2H_3]$ isotopomers of each primary metabolite, leading to exact control of sample recovery. The sensitivity limits are below the subnanomolar levels necessary for quantification of the metabolites formed at sub-K_m concentrations of caffeine, in in vitro incubations.

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