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Letter to the Editor

Radiometric high-performance liquid chromatographic procedure for the determination of theobromine metabolites in microsomal incubations

Sir,

6-Amino-5-(N-methylformylamino)-1-methyluracil (3,7-DAU), 3,7-dimethyluric acid (3,7-DMU), 3-methylxanthine (3-MX), 7-methylxanthine (7-MX) and 7-methyluric acid (7-MU) have been identified as the major metabolites of theobromine (TB; 3,7-dimethylxanthine) in both humans [1, 2] and laboratory animals [3]. Available evidence [1, 2] suggests that cytochrome P-450 is involved in the formation of 3,7-DMU, 3-MX and 7-MX and that xanthine oxidase mediates the secondary conversion of 7-MX to 7-MU. However, the route of formation of 3,7-DAU and the enzyme system(s) involved in the production of this compound remain obscure. To elucidate the mechanism of formation of 3,7-DAU we sought to investigate the effects of various factors known to influence xenobiotic metabolism on TB biotransformation in incubations of rat hepatic microsomes. The high-performance liquid chromatographic (HPLC) procedure used previously [1, 2] for the determination of urinary TB metabolites lacked the sensitivity required for the microsomal incubation studies and it was therefore necessary to develop the radiometric HPLC method described in this communication.

EXPERIMENTAL

Materials

Biochemical co-factors for the microsomal incubations were purchased from Sigma (St. Louis, MO, U.S.A.). TB was obtained from Hamilton Labs. (Adelaide, Australia). 3-MX and 7-MX were purchased from Fluka (Buchs, Switzerland) and 3,7-DMU was purchased from Adams Chemical Co. (Round Lake, IL, U.S.A.). 3,7-DAU was a kind gift from Dr. M.J. Arnaud (La Tour-de-Peilz, Switzerland). [$^{14}C_2$]TB (specific activity 56 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA, U.S.A.). The [$^{14}C_2$]TB was approximately 96% pure and required further purification prior to use in the microsomal incubation studies. This was achieved by injecting multiple 100-μl aliquots of the purchased material onto a μBondapak C_{18} column (30 cm $\times$ 3.9 mm I.D., 10 μm particle size; Waters-Millipore, Sydney, Australia) which was eluted with 8:92 (v/v) aceto-

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nitrile–acetate buffer (10 m*M*, pH 4.2) as the mobile phase. Using a mobile phase flow-rate of 2.0 ml/min the retention time of TB was 4.0 min. The TB peak was collected, pooled and extracted five times with redistilled dichloromethane (solvent-to-column eluent volume ratio, 10:1). The dichloromethane fractions were evaporated to dryness using a rotary evaporator and the [$^{14}C_2$]TB was reconstituted in 5% ethanol–water. Re-injection of this material showed that >99.8% of the radioactivity was associated with the [$^{14}C_2$]TB peak.

Apparatus

The HPLC system used comprised a Waters-Millipore U6K injector, Model 6000 solvent delivery system, Model 450 variable-wavelength detector and Model 730 data module. The chromatograph was fitted with an Altex Ultrasphere ODS column (15 cm×4.6 mm I.D., 5 μm particle size; Beckman Instruments, Sydney, Australia) and operated at room temperature. The mobile phase was 10 m*M* acetate buffer (pH 4.5) at a flow-rate of 2.0 ml/min. Radioactivity was determined using a Beckman Model LS-3801 liquid scintillation counter.

Assay procedure

The incubation mixture (1 ml) routinely contained rat hepatic microsomal protein (1 mg) in Tris–HCl buffer (50 m*M*, pH 7.4), NADPH-generating system (1 m*M* NADP, 10 m*M* glucose-6-phosphate, 2 I.U. glucose-6-phosphate dehydrogenase and 5 m*M* magnesium chloride in Tris–HCl buffer, pH 7.4) and 8.1 nmol of [$^{14}C_2$] (10^6 dpm) plus unlabelled TB to give the desired final concentration. Incubations were carried out at 37°C for 1.5 h. The reaction was stopped by the addition of ammonium sulphate (700 mg) and cooling on ice. The saturated incubation mixture was extracted with 8 ml of dichloromethane–isopropanol (4:1) and the phases were separated by centrifugation (1500 *g* for 5 min). The extraction tube was placed in an acetone–dry ice bath to freeze the aqueous layer (located at the bottom of the tube) and the organic phase was decanted into a clean conical-tip glass tube. This extraction procedure was repeated with the thawed aqueous phase, the organic extracts were combined and then evaporated to dryness. The residue was reconstituted in 0.5 ml of mobile phase to which was added a standard solution of TB metabolites(0.1 ml of a solution containing 1–1.5 mmol/l of each compound). Addition of unlabelled metabolites was necessary to enable visualisation of chromatography fractions using photometric detection at 275 nm. The entire sample was injected onto the chromatograph and fractions corresponding to each metabolite were collected manually into scintillation vials. Following elution of 3,7-DMU, 0.2 ml of acetonitrile was injected onto the column to facilitate the removal of unchanged TB.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram of an extract of a rat hepatic microsomal incubation performed in the presence of [$^{14}C_2$]TB. As indicated in Experimental, unlabelled TB metabolites were added to extracts to enable visualisation of chromatography fractions using UV absorbance detection. Peaks for 3,7-DAU, 7-MX,

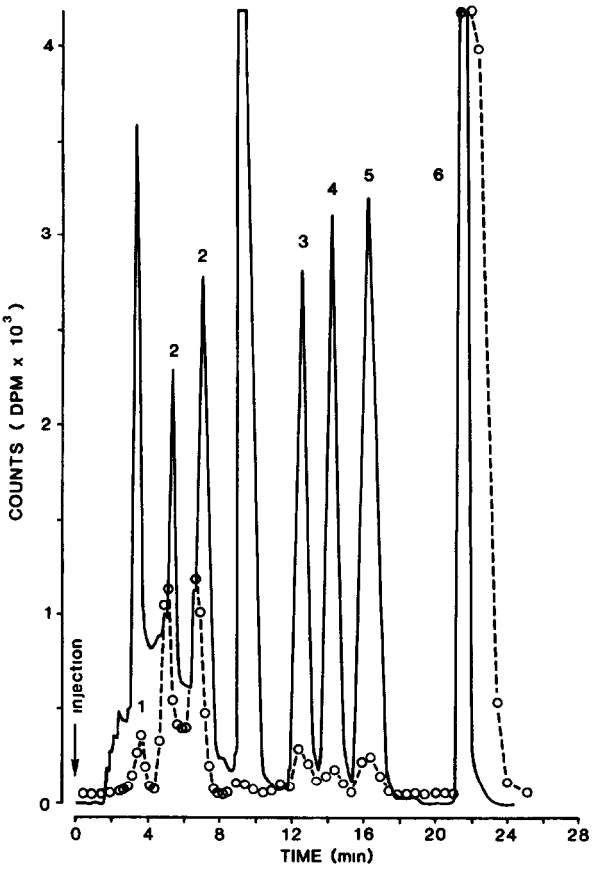

Fig. 1. Chromatogram of [$^{14}C_2$]theobromine products formed in a microsomal incubation containing 2 m*M* glutathione (see Results and discussion). The incubation time was 1.5 h. Solid line indicates the UV absorption (275 nm) of added reference standards and dashed lines with open circles show the radioactivity of labelled metabolites and substrate. Peaks: 1 = unknown polar metabolite; 2 = 3,7-DAU; 3 = 7-MX; 4 = 3-MX; 5 = 3,7-DMU; 6 = TB.

3-MX, 3,7-DMU and TB were well resolved. 3,7-DAU gave peaks at 5.2 and 7.0 min; similar chromatographic behaviour has been reported previously for aqueous solutions of analytically pure 3,7-DAU [1] and for 3,7-DAU excreted in the urine of humans and animals administered TB [1, 2, 4]. Retention times for 7-MX, 3-MX, 3,7-DMU and unchanged TB were 12.8, 14.2, 16.9 and 22.0 min, respectively.

The extraction conditions outlined here resulted in essentially quantitative recovery of the various TB metabolites. Using unlabelled TB and metabolites the recoveries (mean of ten determinations in the concentration range 25–500 μmol/l) of 3,7-DAU, 7-MX, 3-MX, 3,7-DMU and TB were 97 ± 2, 101 ± 3, 98 ± 3, 93 ± 3 and $100 \pm 1\%$. In all experiments using [$^{14}C_2$]TB, $>99.5\%$ of the radioactivity present in the incubation could be accounted for in the combined column fractions. Assay variability was assessed by determining the formation of 3,7-DAU and 3,7-DMU in the same batch of microsomes on a within-day and between-day basis (ten determinations in each case). The mean intra-assay coefficients of

variation for 3,7-DAU and 3,7-DMU formation were 3.9 and 4.1%, respectively, while the mean inter-assay coefficients of variation for 3,7-DAU and 3,7-DMU formation were 7.3 and 8.8%, respectively. Since 3-MXand 7-MX generally only accounted for a small proportion of TB incubation products, it was not possible to measure assay variability for these metabolites.

The assay has been used to demonstrate linearity of 3,7-DAU and 3,7-DMU formation for incubation times and microsomal protein concentration up to 120 min and 2.0 mg/ml, respectively. Moreover, as indicated above it is apparent from preliminary experiments that 3-MX and 7-MX are minor metabolites of TB in rat hepatic microsomal incubations. Normally 3,7-DMU appears to be the major metabolite of TB in microsomal incubations, but when glutathione (2 m*M*) is included in the incubation formation of 3,7-DAU predominates (Fig. 1). This observation is consistent with the result of microsomal incubation studies with caffeine where it was reported [5] that metabolic switching from 1,3,7-trimethyluric acid to the ring-opened uracil 6-amino-5-[N-formylmethylamino]-1,3-dimethyluracil occurred in the presence of glutathione.

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