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Determination of two major metabolites of the novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid in hepatic microsomal incubations by high-performance liquid chromatography with fluorescence detection

Shufeng Zhou[a], James W. Paxton[a,*], Malcolm D. Tingle[a], John McCall[b], Philip Kestell[c]

[a]*Department of Pharmacology and Clinical Pharmacology, The University of Auckland, Auckland, New Zealand*
[b]*Department of Surgery, The University of Auckland, Auckland, New Zealand*
[c]*Auckland Cancer Society Research Center, The University of Auckland, Auckland, New Zealand*

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Abstract

High-performance liquid chromatographic methods have been developed and validated for the glucuronidated and oxidative metabolites of the novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA), produced in human liver microsomal incubations. Calibration curves for DMXAA acyl glucuronide (DMXAA-Glu) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA) were constructed over the concentration ranges of 0.25 to 20 and 0.5 to 40 μM, respectively. Assay performance was determined by intra- and inter-day accuracy and precision of quality control (QC) samples. The difference between the theoretical and measured concentration, and the coefficient of variation, were less than 15% at low QC concentrations, and less than 10% at medium and high QC concentrations for both analytes. The methods presented good accuracy, precision and sensitivity for use in kinetic studies of the glucuronidated and oxidative metabolites of DMXAA in human liver microsomes.

Keywords: 5,6-Dimethylxanthenone-4-acetic acid

1. Introduction

5,6-Dimethylxanthenone-4-acetic acid (DMXAA) is a novel anti-tumour agent currently undergoing phase I clinical trials in New Zealand and the UK (Fig. 1). Unlike conventional cytotoxic anti-cancer agents, DMXAA causes rapid vascular collapse and necrosis in transplantable murine tumours by immune modulation and the induction of cytokines, in particular tumour necrosis factor (TNFα), interferons, nitric oxide and serotonin [1–6]. Studies in rodents, rabbits and humans have indicated that glucuronidation of the acetic acid side chain and 6-methyl hydroxylation are the major metabolic pathways of DMXAA with the resultant metabolites being excreted in bile and urine [7–10]. The two major metabolites of DMXAA have been successfully isolated and purified from human and rat urine using a solid-phase extraction (SPE) method, and identified as DMXAA acyl glucuronide (DMXAA-

*Corresponding author.
E-mail address: j.paxton@auckland.ac.nz (J.W. Paxton)

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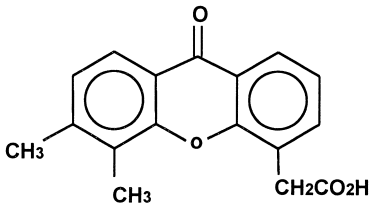
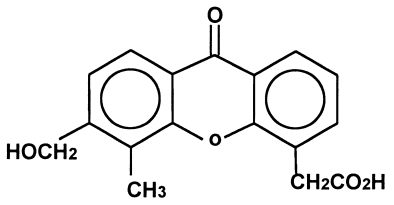
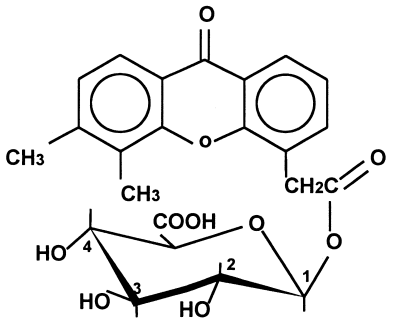


5,6-dimethylxanthenone-4-acetic acid (DMXAA)



6-hydroxymethyl-5-methylxanthenone-4-acetic acid



DMXAA acyl glucuronide

Fig. 1. Chemical structures of DMXAA and its two major metabolites, DMXAA acyl glucuronide and 6-OH-MXAA.

Glu) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA) by mass spectral analysis. Further characterisation of DMXAA metabolism will help to understand the complex pharmacokinetics of DMXAA and predict possible interaction with other drugs, thus rationalising its clinical use and further development.

A high-performance liquid chromatography (HPLC) method with prior SPE has been used for the analysis of DMXAA in plasma and urine for pharmacokinetic studies [10,11]. Studies with isolated perfused rat livers have revealed at least seven metabolites excreted in bile, one of which has been identified by mass spectrometry (MS) as DMXAA-Glu. Another one, resistant to alkaline hydrolysis, was characterised as a hydroxylated DMXAA metabolite [8]. Miners et al. analysed the formation rate of DMXAA-Glu in human liver microsomes by HPLC, but the calibration curve was based on the parent drug, and no internal standard was used [9]. They also did not analyse the oxidative product of DMXAA in human liver microsomes. Previously using HPLC with fluorescence detection, we examined the urine of mouse and rat given a maximum tolerated dose of DMXAA. Two metabolites were observed, which were characterised by MS and ^{1}H-nuclear magnetic resonance to be the acyl glucuronide of DMXAA and 6-OH-MXAA [10]. We report here on HPLC methods with fluorometric detection for the quantitation of two major metabolites, DMXAA-Glu and 6-OH-MXAA, formed in human microsomal incubations.

2. Experimental

2.1. Chemicals

DMXAA and the internal standard, 2,5-dimethylxanthenone-4-acetic acid (SN24350) were synthesised by Dr. G.W. Rewcastle, Auckland Cancer Society Research Centre (ACSRC), The University of Auckland, New Zealand [12]. DMXAA was protected from light exposure to avoid degradation [13]. L-Thalidomide was synthesised by Dr. B. Palmer of ACSRC using the method as described [14]. DMXAA-Glu and 6-OH-MXAA were isolated and purified by a SPE method from human and rat urine, respectively, and their structures identified by MS. Other chemicals were obtained from the following sources: diclofenac, oxazepam, naproxen, cyproheptadine, 1-naphthol, probenecid, amitriptyline, chlorzoxazone, cimetidine, tolbutamide, diethyldithiocarbamate, α-naphthoflavone, sulphaphenazole, troleandomycin, quinidine, D-saccharic acid-1,4-lactone and Brij 58 from Sigma (Sydney, Australia); uridine 5′-diphosphoglucuronic acid (disodium salt; UDPGA) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) from Boehringer Mannheim (Auckland, New Zealand); methanol, acetonitrile, sodium dihydrogenphosphate, disodium hydrogenphosphate, potassium hydroxide, perchloric acid from BDH (Auckland, New Zealand). All other chemicals and reagents were of analytical- or HPLC-grade, as appropriate.

2.2. HPLC instrumentation

The HPLC system consisted of a Model 430 solvent delivery system, an Model SF250 fluorescence detector, a Model 460 autosampler, and a Model D450 data processing system (All from Kontron, Milan, Italy).

A Luna C_{18} guard column was positioned ahead of the 5 μm Spherex analytical column (150×4.6 mm; Phenomenex, Torrance, CA, USA). The mobile phases were acetonitrile–10 m*M* ammonium acetate buffer (24:76, v/v, pH 5.8) and acetonitrile–10 m*M* ammonium acetate (19:81, v/v, pH 5.8) for the analysis of DMXAA-Glu and 6-OH-MXAA, respectively with a flow-rate 1.5 ml/min. These solvents were degassed immediately before use and purged with oxygen-free nitrogen gas during operation. The excitation and emission wavelength of the fluorescence detector were 345 nm and 409 nm, respectively for both DMXAA-Glu and 6-OH-MXAA.

2.3. Microsomal incubation and sample preparation

Human liver microsomes from donors who underwent liver resection for metastasis of colon cancer were prepared by differential centrifugation as describe by Robson [15], and aliquots stored at −80°C until used. Histological examination of the resected livers ensured the use of healthy liver tissue. Microsomal protein concentration was determined by the bicinchoninic acid (BCA) method [16]. Cytochrome P450 contents were measured as described [17]. Ethical approval was obtained from the Northern New Zealand Research Ethics Committee and all donors gave written informed consent for liver tissues to be used for research.

DMXAA glucuronidation in vitro was performed by microsomal incubations of 300 μl containing 0.1 mg/ml human liver microsomal protein, 10 m*M* UDPGA, 5 m*M* $MgCl_2$, 0.1 mg/ml D-saccharic acid-1,4-lactone, 0.05% Brij 58 and DMXAA (5–350 μ*M*) in 0.1 *M* phosphate buffer (pH 6.8). D-Saccharic acid-1,4-lactone was used to inhibit the activity of β-glucuronidase in microsomes. The reaction was initiated by the addition of UDPGA and incubated at 37°C in a water bath with shaking for 20 min. The reaction was stopped by cooling the tubes on ice and adding two volumes of ice-cold acetonitrile–methanol (3:1, v/v) with 2 μ*M* internal standard (SN24350). The tubes containing the mixtures were then centrifuged (3000 *g* for 10 min) to remove precipitated microsomal protein. The supernatant was evaporated using the SpeedVac (Savant Instruments, UK) under nitrogen gas, the residue reconstituted with 300 μl mobile phase, and 50–75 μl was injected onto the HPLC system for measurement of the formation rate of DMXAA-Glu. The formation rate of DMXAA-Glu was calculated as nmol per min per mg microsomal protein (nmol/min/mg). The linearity of the formation rate of DMXAA-Glu was shown with microsomal protein concentration up to 2 mg/ml, and incubation time to 90 min.

The oxidative metabolism of DMXAA in vitro

was performed by microsomal incubations of 200 μl, containing 0.5 mg/ml microsomal protein, 5 m*M* $MgCl_2$, 0.5 m*M* NADPH, and 2.5–80 μ*M* DMXAA in 0.1 *M* phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADPH and conducted at 37°C in a water bath with shaking for 10 min. The reaction was stopped by cooling the tubes on ice and addition of 6 μl 12.1 *M* perchloric acid, and vortex mixing. Then 5 μl 300 μ*M* internal standard SN24350 in 0.1 *M* phosphate buffer (pH 7.4) was added, mixed by vortexing, and then 8 μl 0.5 *M* potassium hydroxide was added to adjust the pH to approximate 5. Each sample was vortexed vigorously for 1 min and centrifuged at 3000 *g* for 10 min. Seventy-five μl of the supernatant was injected onto the HPLC system. The linearity of the formation of 6-OH-MXAA was shown with respect to protein concentration up to 0.5 mg/ml, and incubation time to 20 min under these conditions.

2.4. Calibration curves

Known amounts of DMXAA-Glu or 6-OH-MXAA were added to human liver microsomes separately. Calibration curves were constructed with DMXAA-Glu and 6-OH-MXAA over the concentration range of 0.25 to 20 μ*M* and 0.5 to 40 μ*M*, respectively. Sample preparation of standards was the same as for incubated samples. The ratio of peak area of DMXAA-Glu or 6-OH-MXAA to that of internal standard was graphed against the known concentration, and linear least-squares regression analysis (weighted according to the reciprocal of peak area ratio squared) was conducted to determine the slope, intercept and coefficient of determination by Prizm 2.01 program (Graphpad Software, CA, USA).

2.5. Sensitivity and selectivity

The limit of quantitation (LOQ) was the minimum concentration which could be determined with acceptable accuracy (i.e., recovery between 80 and 120%) and precision [coefficient of variation (CV) $<20\%$] [18]. The limit of detection was defined as the amount which could be detected with a signal-to-noise ratio of 3. The selectivity of the method was examined by determining if interfering chromatographic peaks were present in human liver microsomes from five donors; in microsomal incubations of eight potential inhibitors of UDP-glucuronosyltransferase (UGT) (diclofenac, fenclofenac, 1-naphthol, amitriptylline, oxazepam, cyproheptadine, thalidomide, diflunisal and paracetamol); and in microsomal incubations of nine potential cytochrome P450 inhibitors (chlorzoxazone, cimetidine, diethyldithiocarbamate, ketoconazole, α-naphthoflavone, sulphaphenazole, tolbutamide, troleandomycin, quinidine).

2.6. Accuracy and precision

Quality control (QC) samples containing DMXAA-Glu or 6-OH-MXAA were prepared from weighings, independent of those used for preparing calibration standards. Final concentrations of low, medium and high QC samples were: 0.5, 2.5, 10 μ*M* for DMXAA-Glu; and 1, 5, 20 μ*M* for 6-OH-MXAA. These samples were prepared on the day of analysis in the same way as calibration standards. The performance of the HPLC method was assessed by analysis of 12 QC samples (four each of low, medium and high concentrations) on a single assay day to determine intra-day accuracy and precision, and nine QC samples (three each of low, medium and high concentrations) on each of four consecutive assay days to determine inter-day accuracy and precision.

Extraction efficiency for DMXAA-Glu was assessed at low ($n=3$), medium ($n=3$), and high concentrations ($n=3$) of QC samples. The peak areas of DMXAA-Glu and internal standard SN24350 extracted from the unincubated microsomal mixture were compared to those generated by direct injections of both analytes in mobile phase.

3. Results and discussion

Human liver microsomes were incubated with DMXAA in the presence of UDPGA or NADPH, and the reaction mixture analysed by reversed-phase HPLC. Representative chromatograms from unincubated human liver microsomal mixture with added 5 μ*M* DMXAA acyl glucuronide, incubated human liver microsomes with DMXAA in the absence of

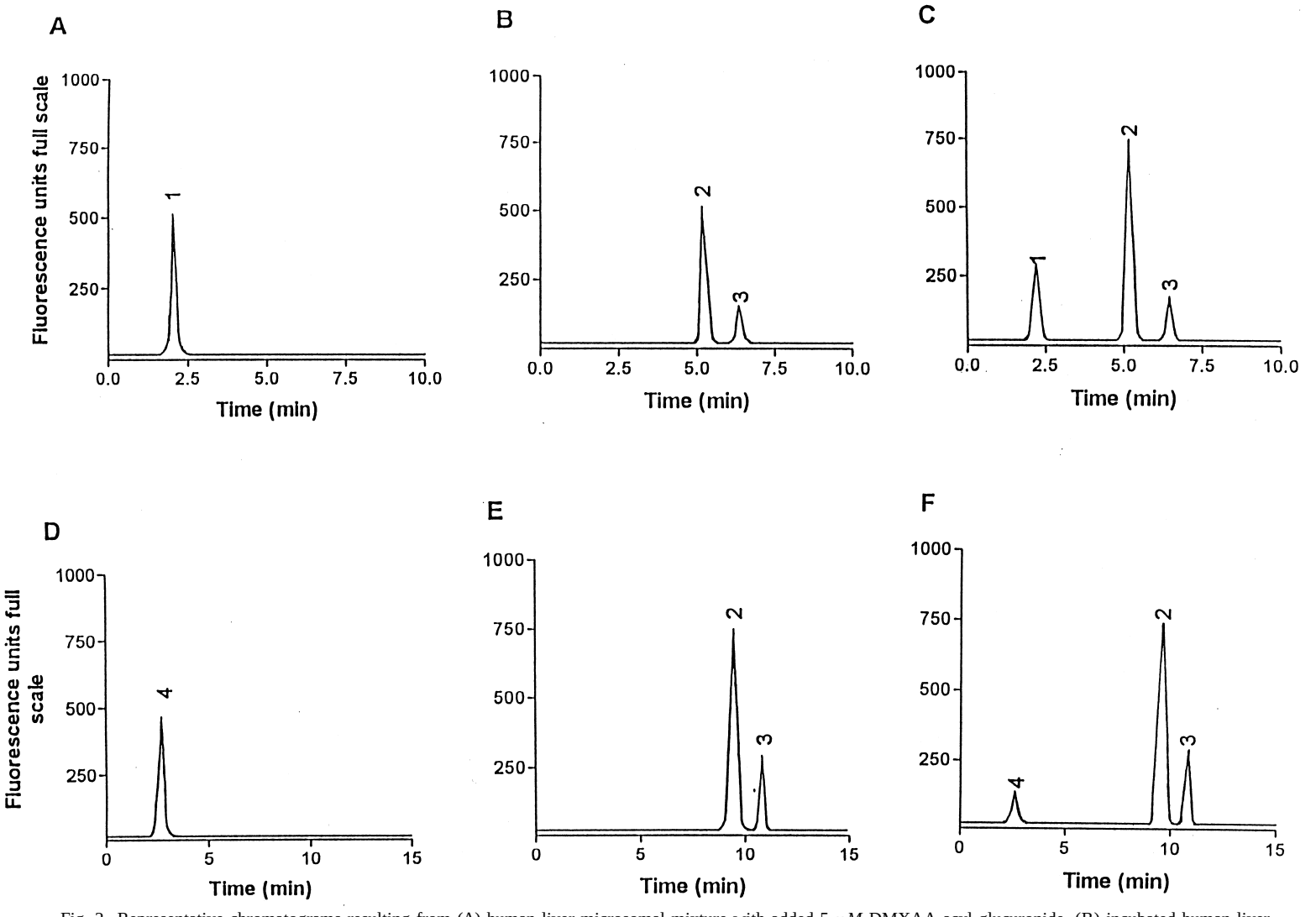


Fig. 2. Representative chromatograms resulting from (A) human liver microsomal mixture with added 5 μ*M* DMXAA acyl glucuronide, (B) incubated human liver microsomal mixture with DMXAA in the absence of UDPGA, (C) incubated human liver microsome with both DMXAA and UDPGA, (D) unincubated human liver microsomal mixture with added 5 μ*M* 6-OH-MXAA, (E) incubated human liver microsomal mixture with DMXAA in the absence of NADPH, (F) incubated human liver microsome with DMXAA and NADPH. Peaks 1 to 4 represent DMXAA-Glu, DMXAA, internal standard SN24350 and 6-OH-MXAA, respectively.

UDPGA, incubated human liver microsomes with both DMXAA and UDPGA, unincubated human liver microsomal mixture with added 5 μ*M* 6-OH-MXAA, incubated human liver microsomes with DMXAA in the absence of NADPH, incubated human liver microsomes with DMXAA and NADPH are shown in Fig. 2. The microsomal incubation with UDPGA produced one major peak that was detected by HPLC with fluorescence with identical retention time to that of authentic DMXAA-Glu. The microsomal incubation with NADPH produced at least three separated major peaks eluting before DMXAA, one of which had an identical retention time to that of authentic 6-OH-MXAA. Under the chromatographic conditions used for the analysis of DMXAA-Glu and 6-OH-MXAA, the retention times for DMXAA-Glu or 6-OH-MXAA, DMXAA and internal standard SN24350 were 2.0, 5.2, 6.3 min, and 2.7, 9.5, 11.5 min, respectively. The total chromatography run time was 8 and 13.5 min for DMXAA glucuronidation and oxidation, respectively.

Extraction efficiencies, expressed as overall mean (±SD) percentage recoveries for DMXAA-Glu (n=9) and internal standard, were 106.1±6.9, 102.4±6.0, respectively. No concentration dependence was observed. DMXAA-Glu is stable under these extraction conditions. It is noteworthy that acyl glucuronides of many acidic drugs are not stable at physiological conditions, undergoing quick hydrolysis to their parent drugs [19]. Furthermore, irreversible binding to proteins can occur via the β-1-*O*-acyl glucuronide and/or via the isomeric conjugate [20–24]. Recently, our laboratory has observed that DMXAA-Glu can be irreversibly bound to human plasma protein in vitro and in vivo [25]. The labile characteristics of acyl glucuronides may confound the metabolic rates of formation determined using traditional methods [26]. True metabolic formation rates in microsomes may be approximated by stabilization of the acyl glucuronide by: (a) a lower incubation pH; (b) use of a β-glucuronidase inhibitor, D-saccharic acid-1,4-lactone; (c) a shorter incubation time [27]. We used pH 6.8 for the glucuronidation incubation and added D-saccharic acid-1,4-lactone to stabilise DMXAA-Glu. Initially the glucuronidation reaction in vitro was halted by addition of phosphoric acid to the incubation mixture, followed by the addition of internal standard and potassium hydroxide, but an interfering peak appeared between DMXAA-Glu and DMXAA in the chromatography. Thus the reaction was stopped by the addition of two volumes of acetonitrile–methanol (3:1, v/v) with internal standard.

Calibration curves were linear over the concentration range used for both analytes with mean r^2 values being greater than 0.999. The differences between the calculated and the true concentrations, and the relative standard deviations were less than 15% at the low QC concentration and less than 10% at medium and high QC concentrations for both metabolites. The accuracy and precision data are

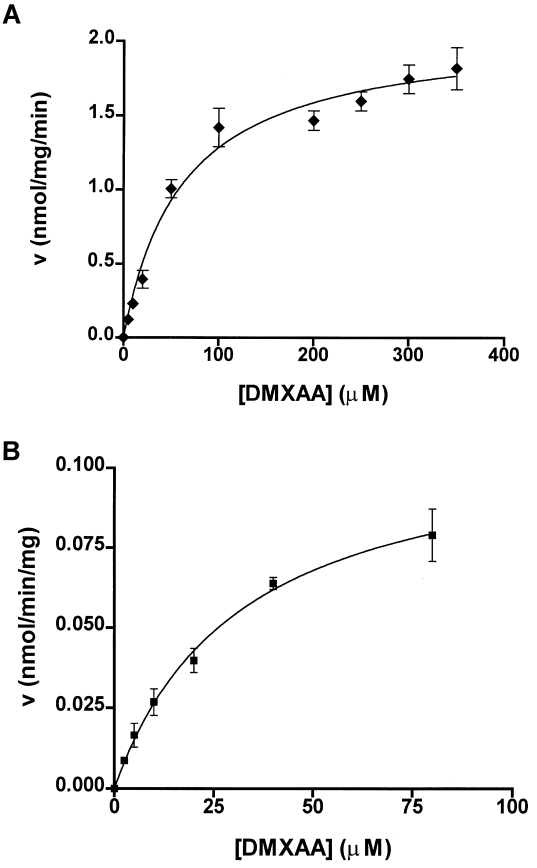


Fig. 3. Typical Michaelis Menten plots for (A) DMXAA glucuronidation in human detergent-activated liver microsomes from donor HL1 and (B) 6-methylhydroxylation of DMXAA in human liver microsomes from donor HL1. Each point represents the mean±SD (n=3).

Table 1
Accuracy and precision of the HPLC methods for the analysis of DMXAA-Glu and 6-OH-MXAA

Theoretical concentration (μM)	Measured concentration (μM, ±SD)	Recovery of theoretical (%)	CV (%)	No. of samples
DMXAA-Glu				
Intra-assay				
0.5	0.510±0.037	101.9	7.3	4
2.5	2.233±0.022	89.3	1.0	4
10	8.944±0.401	89.4	4.5	4
Inter-assay				
0.5	0.488±0.016	97.6	3.4	3
2.5	2.357±0.098	94.3	4.0	3
10	9.451±0.638	94.5	6.2	3
6-OH-MXAA				
Intra-assay				
1	1.084±0.066	108.4	6.1	4
5	5.412±0.415	108.3	7.7	4
20	18.41±0.422	92.0	2.3	4
Inter-assay				
1	0.987±0.105	98.7	12.0	3
5	5.029±0.393	100.6	8.5	3
20	19.96±1.358	99.8	6.5	3

shown in Table 1. The LOQ of the assay was 0.25 and 0.5 μM for a 75 μl injection volume for DMXAA-Glu and 6-OH-MXAA, respectively. In our incubation samples, DMXAA-Glu and 6-OH-MXAA concentrations were always well above the LOQ.

The method was used to perform the kinetic studies of DMXAA glucuronidation and 6-methylhydroxylation in human liver microsomes. Typical Michaelis Menten plots for both metabolites from a donor are illustrated in Fig. 3. Good selectivity of the assay was shown by the absence of interfering chromatographic peaks in microsomal samples from all liver donors and in incubations with potential inhibitors or substrates for UGT and cytochrome P450.

In conclusion, we report HPLC methods for the quantitation of two major metabolites of DMXAA, DMXAA-Glu and 6-OH-MXAA. The HPLC assays for the quantitation of both analytes in human liver microsomal incubations were accurate, reproducible and selective. They have been used to investigate the kinetics and effects of various potential inhibitors on DMXAA glucuronidation and 6-methylhydroxylation in human liver microsomes.

References

[1] L.L. Thomson, L.M. Ching, B.C. Baguley, Cancer Res. 50 (1990) 6966–6970.
[2] A.L. Laws, A.M. Matthew, J.A. Double, M.C. Bibby, Br. J. Cancer 71 (1995) 1204–1209.
[3] M. Philpott, B. C Baguley, L.-M. Ching, Chemother. Pharmacol. 36 (1995) 143–148.
[4] M. Philpott, W.R. Joseph, K.E. Crosier, B.C. Baguley, L.M. Ching, Br. J. Cancer 76 (1997) 1586–1591.
[5] B.C. Baguley, L. Zhuang, P. Kestell, Oncol. Res. 9 (1997) 55–60.
[6] W.R. Joseph, Z. Cao, K.G. Mountjoy, E.S. Marshall, B.C. Baguley, L.M. Ching, Cancer Res. 59 (1999) 633–638.
[7] P. Kestell, G.W. Rewcastle, B.C. Baguley, Xenobiotica 24 (1994) 635–647.
[8] L.K. Webster, A.G. Ellis, P. Kestell, G.W. Rewcastle, Drug. Metab. Dispos. 23 (1995) 363–368.
[9] J.O. Miners, L. Valente, K.J. Lillywhite, P.I. Mackenzie, B. Burchell, B.C. Baguley, P. Kestell, Cancer Res. 57 (1997) 284–289.
[10] P. Kestell, J.W. Paxton, G.W. Rewcastle, I. Dunlop, B.C. Baguley, Cancer Chemother. Pharmacol. 43 (1999) 323–330.
[11] M.J. McKeage, P. Kestell, W.A. Denny, B.C. Baguley, Cancer Chemother. Pharmacol. 28 (1991) 409–413.
[12] G.W. Rewcastle, G.I. Atwell, B.C. Baguley, S.B. Calveley, W.A. Denny, J. Med. Chem. 32 (1989) 793–799.

[13] G.W. Rewcastle, P. Kestell, B.C. Baguley, W.A. Denny, J. Natl. Cancer Inst. 82 (1990) 528–529.
[14] G. Casini, M. Ferappi, Farmaco 19 (1964) 563–565.
[15] R.A. Robson, A.P. Matthews, J.O. Miners, M.E. McManus, U.A. Meyer, P.D. Hall, D.J. Birkett, Br. J. Clin. Pharmacol. 24 (1987) 293–300.
[16] M.G. Redinbaugh, R.B. Turly, Anal. Biochem. 153 (1986) 267–271.
[17] T. Omura, R. Sato, J. Biol. Chem. 239 (1964) 2370–2378.
[18] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249–254.
[19] H. Spahn-Lugguth, L.Z. Benet, Drug Metab. Rev. 24 (1992) 5–48.
[20] P.C. Smith, J.H. Liu, Xenobiotica 23 (1993) 337–348.
[21] N.P. Presle, F. Lapicque, S. Fournel-Gigleux, J. Magdalou, P. Netter, Drug Metab. Dispos. 24 (1996) 1050–1057.
[22] K.A. McGurk, R.P. Remmel, V.P. Hosagrahara, D. Tosh, B. Burchell, Drug Metab. Dispos. 24 (1996) 842–849.
[23] B.C. Sallustio, B.A. Fairchild, P.R. Pannall, Drug Metab. Dispos. 25 (1997) 55–60.
[24] M. Wang, R.G. Dickinson, Drug Metab. Dispos. 26 (1998) 98–104.
[25] S.F. Zhou, J.W. Paxton, P. Kestell, M.D Tingle, 1999, manuscript in preparation.
[26] E.M. Faed, Drug Metab. Rev. 15 (1984) 1213–1249.
[27] H. Spahn-Langguth, L.Z. Benet, Pharmacology 46 (1993) 268–273.