Journal of Chromatography B, 757 (2001) 359–363

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of unbound concentration of the novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid in human plasma by ultrafiltration followed by high-performance liquid chromatography with fluorimetric detection

Shufeng Zhou[a,*], James W. Paxton[a], Malcolm D. Tingle[a], Phillip Kestell[b]

[a]*Department of Pharmacology and Clinical Pharmacology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand*

[b]*Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand*

Received 12 December 2000; received in revised form 20 February 2001; accepted 14 March 2001

Abstract

The novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) is a highly protein bound drug with narrow therapeutic window. We report a simple HPLC method with fluorimetric detection for the determination of free DMXAA concentration in human plasma. Sample preparation involves the ultrafiltration of plasma by a Centrisart device for 30 min at 2000 *g* and extraction with acetonitrile: methanol mixture. The method was validated with respect to recovery, selectivity, linearity, precision, and accuracy. Calibration curves for DMXAA were constructed at the concentration range of 0.5–40 μ*M* in blank plasma and phosphate buffer. The difference between the theoretical and calculated concentration and the relative standard deviation were less than 10% at all quality control (QC) concentrations. The HPLC method has been used for the analysis of preclinical studies. © 2001 Elsevier Science B.V. All rights reserved.

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

1. Introduction

The novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) (Fig. 1) has recently completed a Phase I clinical trial in New Zealand and UK. DMXAA exhibited high plasma protein binding which was concentration-dependent and with significant variation between animal species [1–3]. It has been suggested that the concentration-dependent protein binding may contribute in part to the observed non-linear pharmacokinetics of DMXAA, although other factors relating to its metabolism and

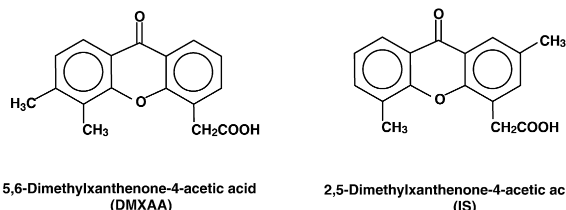

Fig. 1. Chemical structures of DMXAA and the internal standard (I.S.).

*Corresponding author. Tel.: +64-9-373-7599, Ext. 6414; fax: +64-9-373-7556.

E-mail address: shufeng.zhou@auckland.ac.nz (S. Zhou).

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(01)00177-3

biological modifying effects may be more important [3]. These include: (a) its metabolism to form an acyl glucuronide with subsequent hydrolysis to reform parent drug; and (b) its complicated vascular and immune modulatory effects [4,5]. The hydrolysis of acyl glucuronide reforming the parent drug in plasma samples can be significantly reduced by lowering the pH to 5.0. The immune modulatory effects of DMXAA are thought to be responsible for the rapid vascular collapse in the tumour leading to necrosis [4], and the induction of cytokines such as tumour necrosis factor-α, serotonin and nitric oxide [5–10]. In vivo mouse studies have indicated that DMXAA can exert potent anti-tumour activity only at doses that are close to the maximum tolerated doses (30 mg/kg), which results in plasma concentrations of 100–550 μ*M* [3]. The maximum DMXAA concentrations achieved in the plasma of patients in a Phase-I clinical trial ranged from 1000 to 2000 μ*M* [11]. Thus, a greater knowledge of DMXAA's protein binding and its distribution to blood cells may be important in the comparison of anti-tumour/toxicity effects across species, and to achieve optimal results in patients.

In this work we report a validated method for the quantitation of in vitro human plasma free DMXAA concentration using ultrafiltration and a sensitive HPLC method with fluorimetric detection.

2. Experimental

2.1. Chemicals and reagents

The sodium salt of 5,6-dimethylxanthenone-4-acetic acid, 2,5-dimethylxanthenone-4-acetic acid (the internal standard; I.S.) (see Fig. 1) and L-thalidomide were synthesized in the Auckland Cancer Society Research Centre [12,13]. Co-administration of L-thalidomide has been shown to be more effective than D-thalidomide for the potentiation of the anti-tumour activity and increase of the area of plasma concentration–time curve of DMXAA in mice [14]. DMXAA was protected from light exposure to avoid degradation [15]. Diclofenac, cyproheptadine, diazepam and phenylbutazone were purchased from Sigma–Aldrich (Auckland, New Zealand). All other reagents were analytical or HPLC grade as appropriate.

2.2. HPLC instrumentation

The HPLC system consisted of a solvent delivery system, a Model SF250 fluorescence detector (excitation and emission wavelength, 345 and 409 nm, respectively), a Model 460 autosampler, and a Model D450 data processing system (All from Kontron Instrument Co., Milan, Italy). Separation was performed using a 5-μm Spherex C_{18} analytical column (150×4.6 mm) protected by Luna C_{18} guard column from Phenomenex (NZ Ltd., Auckland) and a mobile phase (flow-rate of 2.5 ml/min) of acetonitrile: 10 m*M* ammonium acetate buffer (24:76, v/v, pH 5.0). The solvent was degassed immediately before use and purged with oxygen-free nitrogen gas (Auckland, NZ) during operation.

2.3. Ultrafiltration

Separation of free DMXAA was done by ultrafiltration using the disposable Centrisart micropartition device with 20 000 molecular mass cut-off from Sartorious AG. (Goettingen, Germany). A 0.5-ml plasma sample was divided in two aliquots. A 100-μl aliquot was taken to determine the total DMXAA concentration by HPLC. A 400-μl aliquot of the same sample was then transferred to the ultrafiltration device, centrifuged at 2000 *g* for 30 min at 37°C (Beckman J-6M centrifuge). Samples were capped to minimize changes in pH during filtration. Under these conditions, the ultrafiltration of 0.4 ml plasma results in approximately 150 μl ultrafiltrate. The ultrafiltrate (100 μl) was transferred in a clean glass tube and mixed with 50 μl of 0.1 *M* phosphate buffer (pH 7.4) containing 10 μ*M* I.S., and 50 μl injected into the HPLC.

The adsorption of DMXAA to the Centrisart filtration device was investigated with DMXAA concentrations (0.5, 5, 25, 100, 500 μ*M*) in phosphate buffer. The aqueous solutions were ultrafiltered as described above. The DMXAA concentration in the ultrafiltrate was determined by HPLC. The unbound fraction (f_u) of DMXAA was calculated by

the ratio of the DMXAA concentration in the ultrafiltrate to that in the plasma before ultrafiltration.

2.4. Calibration curves

Quantitation was based on the internal standard method, using the ratio of peak areas and a calibration curve. Calibration curves (0.5–40 μ*M*) were constructed from the peak area ratio of DMXAA: I.S. versus known DMXAA concentrations in plasma or 0.1 *M* phosphate buffer (pH 7.4). Linear least-squares regression analysis was used to determine the slope, intercept and coefficient of determination by Prism 3.0 (Graphpad Software Co., CA, USA). Samples with DMXAA concentrations >40 μ*M* were diluted with 0.1 *M* phosphate buffer (pH 7.4) to ensure that the concentrations were within the assay range.

2.5. Sensitivity and selectivity

The limit of quantitation (LOQ) was determined as the minimum concentration which can be accurately and precisely quantified (in practice it is the lowest data point of the calibration curve), and the limit of detection is defined as the amount which could be detected with a signal-to-noise ratio of 3 [16,17]. The selectivity of the method was examined by determining if interfering chromatographic peaks were present in blank human plasma or in the presence of various drugs including L-thalidomide, diclofenac, diazepam, cyproheptadine, and phenylbutazone. Co-administration of L-thalidomide, diclofenac and cyproheptadine has been shown to reduce the plasma clearance of DMXAA in mouse studies [14,18,19], and the possible plasma protein binding interactions were explored. Diazepam and phenylbutazone were used as known albumin ligands to investigate the binding sites on albumin for DMXAA.

2.6. Accuracy and precision

QC samples containing DMXAA were prepared from weighings independent of those used for preparing calibration curves. Final concentrations of low, medium and high QC samples were 1, 5 and 40 μ*M*. 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 quality control sample (four each of low, medium, and high concentrations) on a single assay day to determine intra-day accuracy and precision, and nine quality control samples (three each of low, medium, and high concentrations) on each of 4 consecutive assay days to determine inter-day accuracy and precision.

2.7. Determination of total plasma DMXAA concentration

Plasma (100 μl) was mixed with 50 μl methanol containing 20 μ*M* I.S., followed by 0.4 ml ice-cold acetonitrile: methanol (3:1, v/v). After centrifugation at 2500 *g* for 15 min to remove precipitated proteins, the supernatant was removed and evaporated to dryness under nitrogen. The residue was dissolved in 200 μl mobile phase, and 50 μl was injected into the HPLC. The calibration curve was constructed using a series of plasma samples spiked with known amounts of DMXAA (0.5–40 μ*M*). Extraction efficiency for DMXAA was assessed at low ($n=3$), medium ($n=3$), and high concentration ($n=3$) of QC samples. The peak areas of DMXAA and internal standard extracted from above procedure were compared to those generated by direct injections of the samples with DMXAA in mobile phase.

2.8. Preclinical application

We used the validated method to study the plasma protein binding of DMXAA in six healthy humans and five patients and the effects of drugs on the protein binding of DMXAA in human plasma. Fresh heparinised blood was obtained from healthy human volunteers ($n=6$) with no known intake of drugs over the previous 4 weeks, and from cancer patients ($n=5$) before the DMXAA infusion during a Phase I trial. All cancer patients had normal renal and hepatic function. Ethical approval was obtained from the Northern New Zealand Research Ethics Commit-

tee and all human subjects gave written informed consent.

3. Results and discussion

Under the chromatographic conditions used for the analysis of DMXAA, the retention times for DMXAA and internal standard were 10.1 and 12.2 min, respectively. The total chromatography run time was 13 min. Matrix-specific interfering peaks that required modification of the mobile phase composition were not observed in any case, particularly when sample work-up included an extraction step or in the presence of drugs such as L-thalidomide and cyproheptadine.

DMXAA concentrations in the pre- and post-centrifuged phosphate buffer were similar, with the ratio ranging from 0.99±0.02 to 1.01±0.03 (n=4), indicating that there was no non-specific binding of DMXAA to the Centrisart devices.

Extraction efficiency for those plasma samples expressed as overall mean (±S.D.) percentage for DMXAA (n=9) and internal standard were 85.1±5.1 and 89.4±4.1 respectively. No concentration dependence was observed. DMXAA is stable under the extraction conditions described.

Calibration curves were linear over the concentration range used with mean correlation coefficients being greater than 0.997 in human plasma and 0.1 M phosphate buffer. The mean y-intercepts for DMXAA were 0.002. The differences between the calculated and the actual concentration and the relative standard deviation were less than 10% at any QC concentrations. The results of the precision and accuracy for protein binding assay were shown in Table 1 and have demonstrated the applicability of the method for the analysis of preclinical studies. The limit of detection of the assay was 0.20 μM for a 75-μl aliquot for DMXAA. The validated method has been used to determine the unbound fraction of DMXAA in human plasma from preclinical studies. The binding of DMXAA in human plasma was concentration-dependent with concentrations ≥1000 μM markedly increasing the f_u of DMXAA (Fig. 2A). The one binding-site model with non-specific binding was the best fit for the binding of DMXAA to human plasma (Fig. 2B).

In conclusion, a sensitive, reliable and accurate HPLC method for the quantitation of unbound DMXAA concentration in human plasma has been

Table 1
Accuracy and precision of the HPLC method for the analysis of unbound DMXAA concentrations in human plasma

Theoretical concentration (μM)	Measured concemtration, mean±S.D.	% Recovery of theoretical	C.V. (%)	No. of samples
0.1 Phosphate buffer				
Intra-assay				
1	0.980±0.067	98.0	6.84	3
5	5.023±0.051	100.5	1.02	3
40	39.24±0.731	98.1	1.86	3
Inter-assay				
1	0.929±0.076	92.9	8.18	4
5	4.917±0.188	98.3	3.82	4
40	40.45±1.908	101.1	2.94	4
Human plasma				
Intra-assay				
1	0.988±0.066	98.8	6.68	3
5	5.012±0.425	100.2	8.48	3
40	39.01±0.911	97.5	2.34	3
Inter-assay				
1	0.944±0.675	94.4	7.15	4
5	5.009±0.311	100.2	6.21	4
40	39.11±1.058	97.8	2.71	4

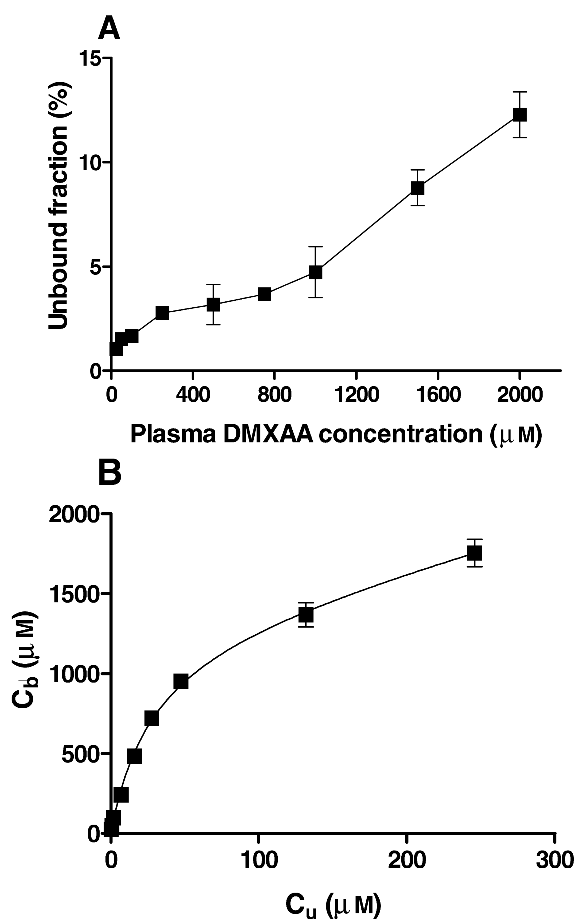

Fig. 2. (A) The relationship between the unbound DMXAA fraction (f_u) and total concentration in human plasma and (B) plot of the plasma bound concentration of DMXAA (C_b) as a function of unbound concentration (C_u) in human plasma; the curves represent the best fit for a one binding-site model with non-specific binding. Human plasma was from one of the six healthy volunteers. Each point represents mean±S.D. of at least three determinations of different samples.

established and validated to investigate the protein binding of DMXAA in preclinical studies.

References

[1] M.J. McKeage, P. Kestell, W.A. Denny, B.C. Baguley, Cancer Chemother. Pharmacol. 28 (1991) 409.
[2] L.K. Webster, A.G. Ellis, P. Kestell, G.W. Rewcastle, Drug Metab. Dispos. 23 (1995) 363.
[3] P. Kestell, J.W. Paxton, G.W. Rewcastle, I. Dunlop, B.C. Baguley, Cancer Chemother. Pharmacol. 43 (1999) 323.
[4] L.J. Zwi, B.C. Baguley, J.B. Gavin, W.R. Wilson, Oncol. Res. 6 (1994) 79.
[5] B.C. Baguley, L. Zhuang, P. Kestell, Oncol. Res. 9 (1997) 55.
[6] L.L. Thomsen, L.-M. Ching, B.C. Baguley, Cancer Res. 50 (1990) 6966.
[7] L.L. Thomsen, L.-M. Ching, L. Zhuang, J.B. Gavin, B.C. Baguley, Cancer Res. 51 (1991) 77.
[8] M. Philpott, B.C. Baguley, L.-M. Ching, Cancer Chemother. Pharmacol. 36 (1995) 143.
[9] M. Philpott, W.R. Joseph, K.E. Crosier, B.C. Baguley, L.-M. Ching, Br. J. Cancer. 76 (1997) 1586.
[10] W.R. Joseph, Z. Cao, K.G. Mountjoy, E.S. Marshall, B.C. Baguley, L.-M. Ching, Cancer. Res. 59 (1999) 633.
[11] M.B. Jameson, P.I. Thomson, B.C. Baguley, B.D. Evans, V.J. Harvey, M.R. McCrystal, P. Kestell, Proc. Annu. Meet. Am. Soc. Clin. Oncol. 19 (2000) 705.
[12] G. Casini, M. Ferappi M, Farmaco 19 (1964) 563.
[13] G.W. Rewcastle, G.I. Atwell, B.C. Baguley, S.B. Calveley, W.A. Denny, J. Med. Chem. 32 (1989) 793.
[14] P. Kestell, L. Zhao, L.-M. Ching, B.C. Baguley, J.W. Paxton, Cancer Chemother. Pharmacol. 46 (2000) 135.
[15] G.W. Rewcastle, P. Kestell, B.C. Baguley, W.A. Denny, J. Natl. Cancer Inst. 82 (1990) 528.
[16] H.T. Kames, G. Shiu, V.P. Shah, Pharm. Res. 8 (1991) 421.
[17] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, Pharm. Res. 9 (1992) 588.
[18] S.F. Zhou, J.W. Paxton, M.D. Tingle, P. Kestell, L.-M. Ching, Cancer Chemother. Pharmacol. (2001) in press.
[19] L. Zhao, P. Ketsell, L. Zhuang, B.C. Baguley, Cancer Chemother. Pharmacol. (2001) in press.