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Determination of the covalent adducts of the novel anti-cancer agent 5,6-dimethylxanthenone-4-acetic acid in biological samples by high-performance liquid chromatography

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*

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Abstract

The reversed-phase HPLC methods were developed to determinate the covalently bound protein adducts of the novel anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA) via its glucuronides after releasing aglycone by alkaline hydrolysis in human plasma and human serum albumin (HSA). An aliquot of 75 μl of the mixture was injected onto a Spherex C_{18} column (150×4.6 mm; 5 μm) at a flow-rate of 2.5 ml/min. The mobile phase comprising of acetonitrile:10 m*M* ammonium acetate buffer (24:76, v/v, pH 5.8) was used in an isocratic condition, and DMXAA was detected by fluorescence. The method was validated with respect to recovery, selectivity, linearity, precision, and accuracy. Calibration curves for DMXAA were constructed in the concentration range of 0.5–40 μ*M* in washed blank human plasma or HSA prior to alkaline hydrolysis. The difference between the theoretical and calculated concentration and the relative standard deviation were less than 10% at all quality control (QC) concentrations. The limit of detection for the covalent adduct in human plasma or HSA is 0.20 μ*M*. The methods presented good accuracy, precision and sensitivity for use in the preclinical and clinical studies.

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

1. Introduction

The novel anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA, Fig. 1) was developed by the Auckland Cancer Society Research Centre (ACSRC). It has recently completed Phase I clinical trials in New Zealand and the UK under the direction of the Cancer Research Campaign's Phase I/II Clinical Trials Committee. As a biological response modifier, DMXAA's mode of action is different from conventional cytotoxic chemotherapeutic agents. In murine models, the anti-tumour activity of DMXAA appears to be mediated by immune modulation and the induction of cytokines, such as tumour necrosis factor [1–3], serotonin [4] and nitric oxide [5,6]. DMXAA is extensively metabolised in animals and humans by glucuronidation on its acetic acid side chain, resulting in DMXAA acyl glucuronide (DMXAA-G), which is excreted into bile and urine [7–9].

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

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

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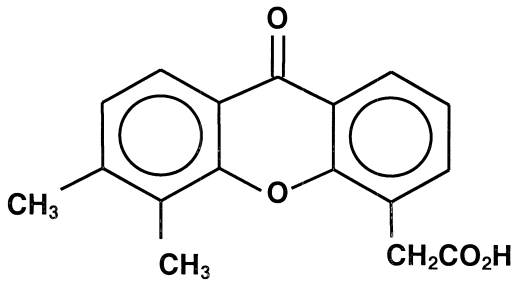

5,6-dimethylxanthenone-4-acetic acid

Fig. 1. Chemical structures of DMXAA.

The acyl glucuronides of many acidic drugs have been shown to be chemically reactive, undergoing hydrolysis, intramolecular rearrangement, and covalent binding to tissue proteins [10–12]. Covalent binding to proteins can be via β1-glucuronides and/or isomers formed by intramolecular migration. The formation of protein adducts have been associated with immune-mediated adverse effects for several drugs such as diclofenac, diflunisal, zomepirac and tolmetin [10,13,14]. Therefore, the study of the reactivity of DMXAA-G has important toxicological implications.

We report here on analytical methods for the determination of the covalently bound protein adducts of DMXAA via its glucuronides after releasing aglycone by alkaline hydrolysis in biological matrixes including human plasma and human serum albumin (HSA) solutions.

2. Experimental

2.1. Chemicals

DMXAA and the internal standard (IS), 2,5-dimethylxanthenone-4-acetic acid (SN24350), were synthesised in the ACSRC [15]. DMXAA was protected from light exposure to avoid degradation [16]. DMXAA-G was isolated and purified by solid-phase extraction method from the urine of patients treated with DMXAA, and its structure identified by liquid chromatography–mass spectrometry and ^{1}H nuclear magnetic resonance [9]. All chemicals were of analytical or HPLC grade as appropriate.

2.2. HPLC instrumentation

The HPLC system consisted of a Model 430 solvent delivery system, a Model SF250 fluorescence detector, a Model 460 autosampler, and a Model D450 data processing system (Kontron, Milan, Italy). A Luna C_{18} guard column was positioned ahead of the 5-μm Spherex analytical column (150× 4.6 mm; Phenomenex, Torrence, CA, USA). The mobile phase (flow-rate of 2.5 ml/min) was acetonitrile:10 m*M* ammonium acetate buffer (24:76, v/v, pH 5.8). The solvent was degassed immediately before use and purged with oxygen-free nitrogen gas (Auckland, NZ) during operation. The excitation and emission wavelength of the fluorescence detector were set at 345 and 409 nm.

2.3. The formation of DMXAA–protein adducts

The in vitro covalent binding of DMXAA via its acyl glucuronide to human plasma and HSA was performed as described [17–19]. To healthy human plasma or HSA (40 g l^{-1}) in 0.1 *M* sodium phosphate buffer at pH 7.4, DMXAA-G (178 μ*M*) was added. After incubation at 37°C for up to 48 h, aliquots (200 μl) were taken at various time to estimate the covalent binding to plasma proteins. Kinetic studies of the covalent binding were performed by incubating DMXAA-G (8.9–712 μ*M*) and 40 g l^{-1} HSA in 0.1 *M* phosphate buffer (pH 7.4) at 37°C for up to 6 h.

Blood samples were taken at 0, 0.083, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12 and 24 h from two cancer patients following a DMXAA dose of 2600 mg m^{-2} by intravenous injection over 20 min. Blood samples were immediately centrifuged, the plasma separated and DMXAA-G was stabilised by the addition of 42.5% orthophosphoric acid (10 μl ml^{-1} of plasma), and stored at −20°C until assayed for the plasma DMXAA–protein adducts.

2.4. Sample preparation

Human plasma or HSA solutions (200 μl) containing the DMXAA–protein adducts were mixed with 2 volumes of ice-cold acetonitrile:methanol (3:1, v/v) containing 1% (v/v) acetic acid and 10 μ*M* IS. After cooling on ice to precipitate the proteins, the pH was adjusted to approximately 5 with fresh 1 *M* KOH and centrifuged at 1500 *g* for 10 min. The precipitated protein pellet was washed 12 times by vigorous voltexing for 2 min with 6 volumes of methanol:diethylether (3:1, v/v), and dried at 40°C under nitrogen flow using a Speedvac solvent concentrator (Solvant Instruments, UK). In preliminary experiments, methanol:acetonitrile (3:1, v/v) and acetonitrile:diethylether (3:1) have been investigated and compared with methanol:diethylether (3:1, v/v) for their protein pellet washing efficiency. The latter gave the highest extraction efficiency and least washing times ($n=12$). HPLC analysis of the final washes ensured that there was no unbound DMXAA or DMXAA-G remaining in the pellet. The dried protein was hydrolysed by addition of 1 *M* KOH (400 μl) and incubation at 70°C for 2.5 h. After hydrolysis, aliquots (20 μl) of the incubate were added to test tubes with 380 μl 1*M* NaOH and stored at −20°C for protein assays by the BCA method using bovine serum albumin as standard [20]. The remaining incubate was acidified to approximately pH 3 by the addition of 42.5% phosphoric acid (38 μl), followed by 500 μ*M* internal standard in 0.1 phosphate buffer (pH 5.0) (10 μl), and the mixture extracted with 2 ml dichloromethane to remove the DMXAA released by hydrolysis. After gentle shaking for 5 min, the tubes were centrifuged at 3000 *g* for 5 min. The aqueous phase was then discarded and the organic phase was evaporated to dryness at 40°C under nitrogen. The residue was reconstituted with 200 μl mobile phase and the DMXAA determined by HPLC. The extent of covalent binding was normalised to protein concentration and expressed as the amount of DMXAA (in ng) bound per mg of protein. Adduct loss was determined by the calculation from protein concentrations in untreated plasma and after the washing procedure. DMXAA that was not removed by the washing procedure, but released only on treatment of the protein precipitate with strong base, was defined as ''covalently bound'' [21].

2.5. Calibration curves

Calibration standards were set up over the concentration range of 0.5–40 μ*M* by adding known amounts of DMXAA to washed blank human plasma or HSA solution prior to alkaline hydrolysis. Sample preparation of standards was the same as for incubated samples. The ratio of peak area of DMXAA to that of internal standard, and linear least-squares regression analysis weighted according to the reciprocal of peak area ration squares was conducted to determine the slope, intercept and coefficient of determination by Prism 3.0 (Graphpad Software, CA, USA).

2.6. 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 [22,23]. The selectivity of the method was examined by determining if interfering chromatographic peaks were present in blank human plasma or HSA.

2.7. 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 four consecutive assay days to determine inter-day accuracy and precision.

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 the covalent binding assay were compared to those generated by direct injections of the samples with DMXAA in mobile phase.

3. Results and discussion

Representative chromatograms from human plasma with and without added DMXAA-G are shown in Fig. 2A,B, respectively. The plasma incubation with DMXAA-G produced one major peak that was detected by HPLC with fluorescence with identical retention time to that of authentic DMXAA. Under the chromatographic conditions used for the analysis of DMXAA, the retention times for DMXAA and internal standard were 10.2 and 12.1 min, respectively. The total chromatography run time was 13.5 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.

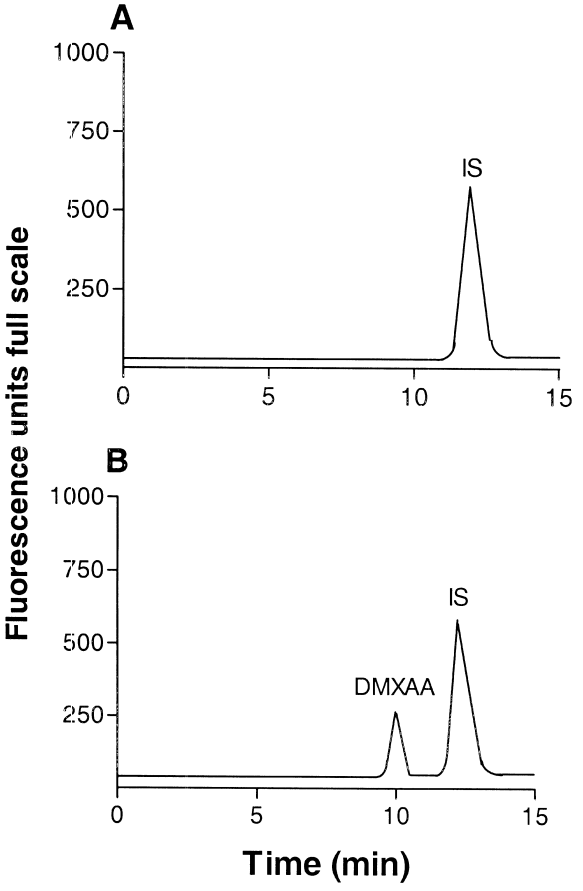

Fig. 2. Representative chromatograms resulting from (A) healthy blank human plasma without added DMXAA acyl glucuronide, (B) healthy human plasma with added 350 μ*M* DMXAA acyl glucuronide. I.S., the internal standard SN24350. The samples were extensively washed and then hydrolysed by strong alkaline.

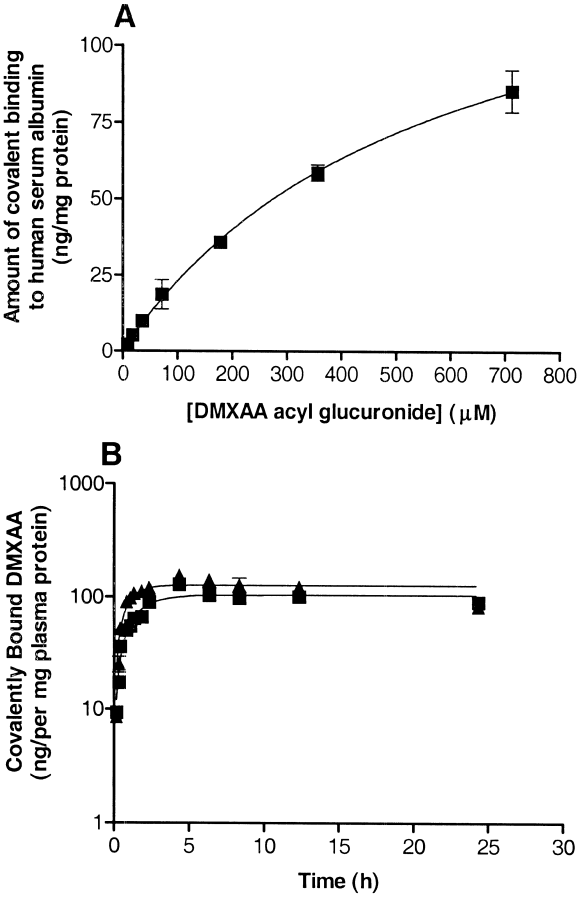

Fig. 3. (A) Michaelis–Menten plot for the formation of DMXAA–protein adducts in human serum albumin (40 gl^{-1}). The curve represents the fitting line of one binding site model with a K_d and N value of 553±37 and 151±6 μ*M*, respectively. (B) The plasma concentration–time profile for DMXAA–protein adduct in two cancer patients receiving DMXAA treatment. A one-compartment model with zero order input and first-order decay was fitted, resulting in a k value of 0.75 and 1.23 h^{-1} for the two patients, respectively.

Extraction efficiency expressed as overall mean (±SD) percentage for DMXAA (n=9) and internal standard were 90.1±5.9 and 91.4±4.7, respectively. No concentration dependence was observed. DMXAA is stable under the extraction conditions described. DMXAA, DMXAA-G and adducts were stable in acidified plasma and HSA samples. The efficacy of protein washing procedure in removing non-covalently bound aglycone and loss of adduct and protein were studied. Control studies with DMXAA, as well as HPLC analysis of washes, indicated that the washing procedure completely removed adsorbed and reversibly bound DMXAA from protein precipitates after 12 times washes. The mean adduct loss due to protein washing was 15.8±1.8% (n=58), which was accounted for by normalization of adduct concentrations on the basis of the residual protein fraction. Calibration curves were linear over the concentration range used with mean correlation coefficients being greater than 0.994 (±0.002) (n=5). The mean y-intercepts for DMXAA were 0.004 (±0.001) (n=5). 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 covalent binding assay were shown in Table 1 and have demonstrated the applicability of the methods for the analysis of preclinical and clinical studies. The limit of detection of the assay (the minimum concentration which could be determined with acceptable accuracy (20%), and precision (C.V.<20%)) was 0.20 μM for a 75-μl aliquot for DMXAA. The validated method has been used to determine the formation of DMXAA–protein adduct from preclinical and clinical studies. The Michaelis–Menten plot for the covalent binding of DMXAA-G to HSA and the formation of DMXAA–protein adducts in plasma over 24 h after DMXAA administration in two patients are shown in Fig. 3A,B, respectively. The in vitro formation of DMXAA–protein adducts followed Michaelis–Menten kinetics, and one binding site model was the best one with a K_d value of 553±37 μM and an N value of 151±6 μM. K_d is the apparent dissociation constant for the binding site; N is the concentration of binding sites on plasma protein. To analyse in vivo DMXAA–protein adduct formation data, it was assumed that the rate of

Table 1
Accuracy and precision of the HPLC methods for the analysis of DMXAA–protein adducts in human plasma and albumin solution

Theoretical conc. (μM)	Measured conc. Mean±SD	% Recovery of theoretical	C.V. (%)	No. of samples
Human plasma				
Intra-assay				
1	0.910±0.047	91.0	5.16	3
5	5.033±0.052	100.7	1.03	3
40	38.94±0.811	97.4	2.08	3
Inter-assay				
1	0.888±0.066	88.8	7.43	4
5	4.857±0.198	97.1	4.08	4
40	39.45±1.938	98.6	4.91	4
HSA				
Intra-assay				
1	1.081±0.076	108.1	7.03	3
5	5.112±0.405	102.2	7.92	3
40	38.41±0.922	96.1	2.40	3
Inter-assay				
1	0.937±0.855	93.7	9.12	4
5	5.129±0.291	102.6	5.67	4
40	39.06±1.958	97.7	5.01	4

adduct formation would approximate a zero-order process at the concentrations of DMXAA-G achieved. Therefore, the in vivo DMXAA–protein adduct formation was fitted to the simple one-compartment pharmacokinetic model assuming zero-order input and first-order elimination, resulting in elimination constant (k) values of 0.75 and 1.23 h^{-1} for the two patients, respectively.

The covalent binding of acyl glucuronide to albumin protein may involve two mechanisms: transacylation and glycation [11,18,19]. Transacylation mechanism involves nucleophilic attack of functional groups ($-NH_2$, –SH, –OH) on proteins at the acyl carbonyl of the β-1-glucuronide or its rearrangement isomers, whilst the glycation mechanism involves intramolecular rearrangement allowing formation of an open-chain conjugate with a free aldehyde group to react with amino group on proteins resulting in adduct formation. Other proteins such as hepatic functional enzymes may also be the targets of acyl glucuronides. To understand the consequences of adduct formation, it is necessary to identify the target protein of covalent binding, the subcellular localization and kinetics of adduct formation and clearance. Thus, different binding mechanisms and adduct types are required to be considered with respect to alternative adduct assays.

In conclusion, a sensitive, reliable and accurate HPLC method for the quantitation of DMXAA protein adducts has been established and validated to investigate the covalent protein adducts in preclinical and clinical studies.

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