Journal of Chromatography, 564 (1991) 315–321
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5673

Short Communication

Determination of xanthenone-4-acetic acid in mouse plasma by high-performance liquid chromatography

P. KESTELL*, M. J. McKEAGE and B. C. BAGULEY

Cancer Research Laboratory, University of Auckland Medical School, Private Bag, Auckland (New Zealand)

(First received July 31st, 1990; revised manuscript received October 9th, 1990)

ABSTRACT

Xanthenone-4-acetic acid (XAA) was synthesised during a search for improved analogues of flavone-8-acetic acid, an antitumour agent with a unique mechanism of action but with a number of pharmacological disadvantages. We describe a simple, selective high-performance liquid chromatographic assay suitable for the detection of XAA in mouse plasma. After addition of an internal standard (3-methyl-XAA), plasma was acidified with trichloroacetic acid and extracted with toluene. After evaporation of solvent, samples were chromatographed on a C_{18} 4-μm Novapak cartridge (mobile phase: water–acetonitrile–acetic acid, 65:35:2, v/v) using fluorescence detection. At the maximum tolerated dose of XAA (725 μmol/kg), non-linear pharmacokinetics were observed.

INTRODUCTION

Xanthenone-4-acetic acid (XAA, Fig. 1) is a fused-ring analogue of the novel anticancer agent flavone-8-acetic acid (FAA, Fig. 1), a synthetic flavonoid which has undergone extensive phase I and II clinical trials [1]. The selection of FAA for testing in man was based upon the unique spectrum of activity found in preclinical screening. FAA is particularly active against slow growing solid tumours in mice such as colon 38 adenocarcinoma and lacks the usual toxicities associated with cytotoxic drugs [2,3]. However, clinically FAA was most disappointing in that it was inactive and exhibited dose-dependent pharmacokinetics [4–6], a phenomena previously reported in mice [7,8]. Despite this, there is still much interest in FAA because its anti-tumour properties arise from mechanisms which are unlike those encountered for compounds used in conventional cancer chemotherapy [9]. It is believed that understanding these mechanisms may lead to a new class of anti-tumour compounds.

Recently the development of more potent analogues of FAA with desirable pharmacokinetics has been undertaken. In our laboratory this has resulted in the

0378-4347/91/$03.50

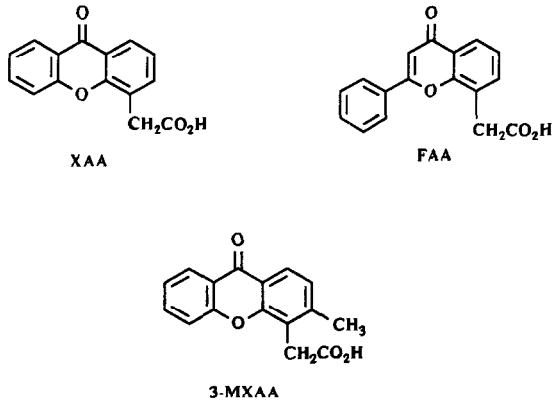

Fig. 1. Structures of xanthenone-4-acetic acid (XAA), flavone-8-acetic acid (FAA) and 3-methylxanthenone-4-acetic acid (3-MXAA, internal standard).

identification of a series of derivatives of XAA. These compounds have similar biological properties to FAA in that they induce tumour haemorrhagic necrosis and elicit growth delays in colon 38 tumours [10]. The parent compound XAA was initially selected from this series for further investigation. Of foremost interest was whether the pharmacokinetics of XAA differ from those of FAA. The quantitation of XAA in biological fluids has not been reported, but there are several high-performance liquid chromatographic (HPLC) methods for the determination of FAA and related compounds in plasma and urine of humans and mice using both liquid–liquid [5,7,8,11] and solid-phase [12,13] extraction techniques. In assessing their applicability to XAA quantitation, it appeared that liquid–liquid extraction was the easier method with our available laboratory apparatus. This report describes a simple and selective HPLC assay suitable for the determination of XAA in mouse plasma and its application to pharmacokinetic studies.

EXPERIMENTAL

Chemicals and standard solutions

XAA and the internal standard, 3-methylxanthenone-4-acetic acid (3-MXAA, Fig. 1), were synthesised as their sodium salts according to published methods [10] and standard solutions of these compounds were prepared by dissolving accurately weighed samples in Millipore Milli-Q water (XAA, 2000 μM; 3-MXAA, 100 μM). Once prepared, the solutions were stored in the dark at room temperature and used within one week. Toluene, acetonitrile and acetic acid were of HPLC grade and trichloroacetic acid was of analytical grade.

Calibration curves and extraction procedure

Working solutions of XAA were made by diluting the standard solution with water and aliquots of these were added to blank mouse plasma to give concentrations ranging from 4 to 20 μM. Aliquots (100 μl) of the resulting plasma solutions were transferred to 13 × 100 mm glass culture tubes containing 0.5% (w/v) trichloroacetic acid (1 ml) followed by 10 μl of the standard solution of 3-MXAA. Toluene (5 ml) was then added as the extracting solvent and the tubes shaken for 30 min. The organic layer was removed by Pasteur pipette to 13 x 75 mm glass tubes and the solvent evaporated using a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). After evaporation, the residue was reconstituted with 100 μl of mobile phase of which aliquots (40 μl) were injected into the chromatograph.

Apparatus and chromatographic conditions

The HPLC system consisted of a Waters 6000A pump and Z module fitted with a C_{18}, 4 μm, 10 cm × 8 mm I.D., Novapak cartridge (Waters Assoc., Milford, MA, U.S.A.), a Model RF-530 Shimadzu fluorescence detector (Shimadzu, Kyoto, Japan) and a Gilson Model 231-401 auto-sampling injector (Gilson Medical Electronics, Middleton, WI, U.S.A.). The excitation and emission wavelengths of the fluorescence detector were set at 345 and 409 nm, respectively. The mobile phase consisted of water–acetonitrile–acetic acid (65:35:2, v/v) and was pumped at a flow-rate of 1 ml/min. Data acquisition and integration was performed using a Philips PU6000 chromatography data system (Philips Analytical, Cambridge, U.K.).

Quantitation

Calibration curves were constructed by plotting peak-height ratios of XAA to the internal standard against concentrations of XAA. Equations for the best-fit straight lines were determined by linear regression analysis. Quantitation of XAA in unknown samples was achieved by calculating the peak-height ratio in the unknown sample and using the equation obtained from the linear regression to calculate a concentration.

Pharmacokinetic parameters

The pharmacokinetic parameters were calculated by a non-compartmental method based on statistical moment theory [14]. The area under the concentration–time curve (AUC_0^∞) was computed using the trapezoidal rule while successive concentration values were increasing, and the log trapezoidal rule while successive concentration values were decreasing after the maximum, and extrapolated to infinity. The area under the moment curve ($AUMC_0^\infty$) was calculated in a similar fashion to the AUC_0^∞ and represented the total area under the first moment of the concentration curve. The mean residence time (MRT) was computed by dividing the $AUMC_0^\infty$ by the AUC_0^∞, and was a measure of the

average time the parent compound remained in the body. The apparent volume of distribution at steady state (V_{ss}) was determined by dividing the product of the dose and $AUMC_0^\infty$, by the AUC_0^∞. Plasma clearance (Cl) was calculated by dividing the dose by the AUC_0^∞.

RESULTS AND DISCUSSION

The best results were achieved after extracting plasma samples with toluene. Fig. 2 depicts chromatograms corresponding to extracts of control mouse plasma (A), control mouse plasma to which XAA and internal standard had been added (B) and mouse plasma after intravenous administration of XAA (C). The retention times for XAA and internal standard were 7.2 and 9.2 min, respectively, and these remained virtually constant throughout an analytical run. The plasma toluene extracts did not contain any interfering peaks by fluorescence detection. Such solvents as dichloromethane, dichloroethane, ethyl acetate and diethyl ether were tested but found unsuitable as the resulting extracts either contained endogenous compounds that co-eluted with XAA and the internal standard, or the xanthenones were extracted in low yields. Toluene also had the advantage in that it selectively extracted XAA and not hydroxylated XAAs which are possible metabolites of XAA. The absolute recoveries of XAA from mouse plasma with toluene extraction were determined by comparing the peak heights obtained from direct injections of the working solutions of XAA with those obtained from plasma extracts to which the same concentration of XAA had been added. Absolute recoveries varied from 79.6 to 84.6% over a 4–20 μM concentration range.

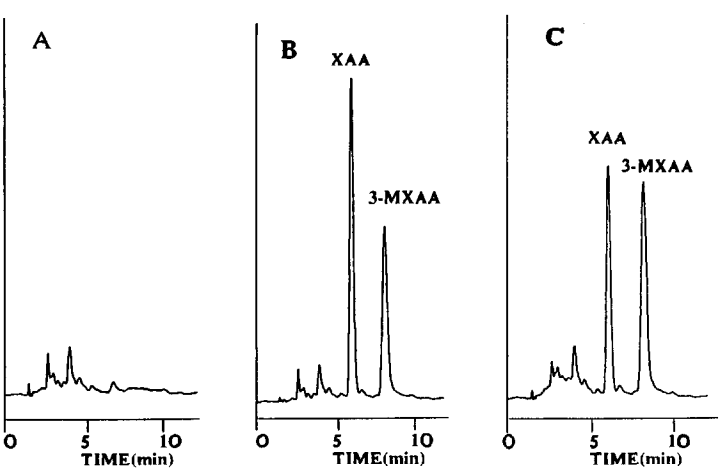

Fig. 2. Representative chromatograms of plasma extracts obtained from (A) control mouse plasma, (B) control mouse plasma with XAA (20 μM) and internal standard (10 μM) added and (C) plasma sampled from a mouse 48 h after intravenous administration of XAA at 725 μmol/kg.

The results for the intra- and inter-day variation of the method are given in Table I. The intra-day precision was determined after analysing plasma samples to which XAA had been added in the concentration range 4–20 μM. In a single run, eight separate plasma samples were prepared at 4, 8, 12, 16 and 20 μM. These were extracted and analysed on the same day. For the inter-day variation, three separate plasma samples at the same concentrations were prepared and analysed, and this procedure was repeated on three consecutive days. The coefficients of variation (C.V.) for the intra-day precision of XAA ranged from 1.77 to 4.63% and that for the inter-day variation ranged from 1.22 to 9.56%. The sensitivity limit of the assay as determined by a signal-to-noise ratio of 3:1 was 0.2 μM for a 100-μl sample. This could be improved to 0.08 μM if the sample size was increased to 250 μl. Calibration plots of peak-height ratio *versus* XAA concentration were linear over the concentration range 4–20 μM and the following general equation was obtained by regression analysis: $y = 0.857x + 0.05$. The corresponding correlation coefficients for the calibration plots varied between 0.991 and 0.998. Stability studies were performed on plasma samples containing XAA at concentrations of 4 and 20 μM stored at -80°C for two months and analysed weekly. During this period the concentration of XAA changed very little (20.6 ± 1.08, 3.76 ± 0.2 μM) indicating that the compound was stable in plasma when stored under these conditions.

Care was required when working with solutions prepared from the sodium salts of either XAA or 3-MXAA as both these compounds are known to undergo decomposition by photolytic decarboxylation [15]. Therefore all procedures involving these solutions and plasma samples containing XAA were carried out in subdued light. If such precautions were not taken, photolytic decomposition of

TABLE I

PRECISION OF THE ASSAY OF XAA IN MOUSE PLASMA

	Concentration added (μM)	Mean concentration measured (μM)	C.V. (%)
Intra-day	20	19.9	4.06
($n = 8$)	16	16.8	1.77
	12	12.3	2.57
	8	7.92	3.78
	4	4.14	4.63
Interday	20	20.5	3.61
($n = 9$)	16	18.1	1.22
	12	12.8	2.89
	8	8.14	3.23
	4	3.98	9.56

XAA resulted in the formation of 4-methylxanthenone (retention time 1.2 h) which appeared in the later chromatograms during an analytical run as a broad shaped peak that interfered with precise measurement of peak heights thus rendering the analysis unreliable. Furthermore decomposition of XAA in the standard solutions resulted in large errors in the calibration graph.

The assay has been applied to the determination of XAA in mouse plasma following intravenous administration of XAA to male BDF_1 mice bearing colon 38 tumours at 725 μmol/kg (200 mg/kg). The resulting concentration–time profile is given in Fig. 3 and indicates non-linear kinetics at this dose in mice. The following model-independent pharmacokinetic parameters were calculated: C_{max} (maximum concentration measured), 1776 μM; MRT, 11.3 h; AUC_0^∞, 22 578 μmol · h/l; Cl, 0.032 l/h/kg; V_{ss}, 0.345 l/kg. They were compared with those obtained for FAA at a similar dose level [8]. The results suggest that pharmacokinetic differences do occur between XAA and FAA as reflected in the longer half-life, greater AUC and slower clearance. It is hoped that the HPLC method developed for XAA would be adaptable for the quantitation of other more potent XAA analogues in mouse plasma and that their pharmacokinetics could be investigated in order to compare them with XAA and FAA.

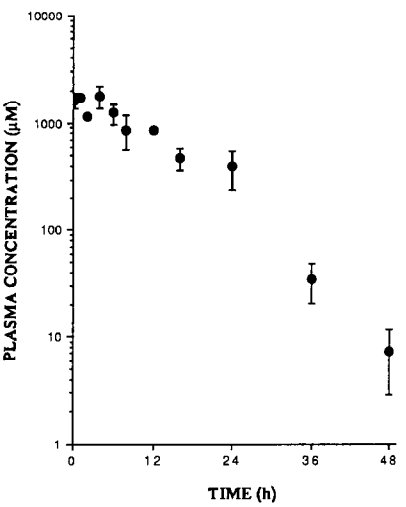

Fig. 3. Plasma XAA concentrations after intravenous administration to male BDF_1 mice bearing colon 38 tumours at 725 μmol/kg. Each point is the mean ± S.D. for three mice.

ACKNOWLEDGEMENTS

This work was supported by the Auckland Division of the Cancer Society of New Zealand, the Todd Foundation and a Warner-Lambert Fellowship. The authors also thank Lynden Wallis for typing the manuscript.

REFERENCES

1 J. D. Kerr and S. B. Kaye, *Eur. J. Cancer Clin. Oncol.*, 32 (1989) 1271.
2 J. Plowman, V. L. Narayanan, D. Dykes, E. Szarvasi, P. Briet, O. C. Yoder and K. Paull, *Cancer Treat. Rep.*, 70 (1986) 631.
3 D. S. Zaharko, C. K. Grieshaber, J. Plowman and J. C. Cradock, *Cancer Treat. Rep.*, 70 (1986) 1415.
4 D. J. Kerr, T. Maughan, E. Newlands, G. Rustin, N. M. Bleehen, C. Lewis and S. B. Kaye, *Br. J. Cancer*, 60 (1989) 104.
5 D. J. Kerr, S. B. Kaye, J. Cassidy, C. Bradley, E. M. Rankin, L. Adams, A. Setanoians, T. Young, G. Forrest, M. Soukop and M. Clavel, *Cancer Res.*, 47 (1987) 6776.
6 R. B. Weiss, R. F. Greene, R. D. Knight, J. M. Collins, J. J. Pelosi, A. Sulkes and G. A. Curt, *Cancer Res.*, 48 (1988) 5878.
7 G. Damia, M. L. Aanette, C. Rossi, R. Mandelli, A. Ferrari and M. D'Incalci, *Cancer Chemother. Pharmacol.*, 22 (1988) 47.
8 G. G. Chabot, M.-C. Bissery, T. H. Corbett, K. Rutkowski and L. H. Baker, *Cancer Chemother. Pharmacol.*, 24 (1989) 15.
9 J. Cummings and J. F. Smyth, *Cancer Chemother. Pharmacol.*, 24 (1989) 269.
10 G. W. Rewcastle, G. J. Atwell, B. C. Baguley, S. B. Calveley and W. A. Denny, *J. Med. Chem.*, 32 (1989) 793.
11 L. Zecca, L. Guadagni and S. R. Bareggi, *J. Chromatogr.*, 230 (1982) 168.
12 J. A. Double, M. C. Bibby and P. M. Loadman, *Br. J. Cancer*, 54 (1986) 595.
13 J. Cummings, D. J. Kerr, S. B. Kaye and J. F. Smyth, *J. Chromatogr.*, 431 (1988) 77.
14 M. Gibaldi and D. Perrier, *Pharmacokinetics*, Marcel Dekker, New York, 2nd ed., 1982, Ch. 11, p. 409.
15 G. W. Rewcastle, P. Kestell, B. C. Baguley and W. A. Denny, *J. Natl. Can. Inst.*, 82 (1990) 528.