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High-performance liquid chromatographic method for sensitive determination of the alkylating agent CB1954 in human plasma

D. Anderson[a], D.R. Ferry[a], R.J. Knox[b], S.J. Andrews[a], A.J. Downes[a], D.J. Kerr[a], L.W. Seymour[a,*]

[a]*CRC Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TA, UK*
[b]*Enzacta Ltd, Porton Down Science Park, Salisbury SP4 0JQ, UK*

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Abstract

A high-performance liquid chromatography (HPLC) method is described for the measurement of the weak alkylating agent CB1954 in human plasma. CB1954 can be used as an innocuous prodrug designed for activation by bacterial nitroreductases in strategies of gene-directed enzyme–prodrug therapy, and becomes activated to a potent bifunctional alkylating agent. The HPLC method involves precipitation and solvent extraction and uses Mitomycin C (MMC) as an internal standard, with a retention time for MMC of 5.85±0.015 min, and for CB1954 of 10.72±0.063 min. The limit of detection for CB1954 is 2.9 ng/ml, and this compares favourably with systems involving direct analysis of plasma (limit of detection 600 ng/ml, approximately). The method is now being used for pharmacokinetic measurements in plasma samples from cancer patients entering phase I clinical trials of CB1954. Results using serial plasma samples from one patient are presented. The patient was treated intravenously with CB1954 (6 mg/m^2), and plasma clearance of the drug showed biphasic kinetics with α half-life 14.6 min, and β half-life 170.5 min. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: CB1954; Alkylating agents

1. Introduction

Cytotoxic chemotherapy based on gene therapy encompasses the concept of gene-directed enzyme–prodrug therapy (GDEPT) [1]. In this approach, transgenes are delivered that encode enzymes capable of in situ activation of relatively innocuous prodrugs to yield potent cytotoxic species [2]. If the transgenes can be expressed selectively within appropriate tumour cells in vivo, the prodrug can then be administered systemically and cytotoxicity will be restricted to sites of drug activation, i.e., sites of transgene expression within the tumour. One enzyme attracting particular attention in this context is the *Escherichia coli* nitroreductase, capable of activating several useful prodrugs, including the weak monofunctional-alkylating agent CB1954 (Fig. 1) [3–8]. Nitroreductase activation of CB1954 results in conversion of the 4-nitro group into a hydroxylamine derivative, and the resulting 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is modified intracellularly to yield species with potent bifunctional alkylating activity, capable of crosslinking DNA and leading to irreparable genomic damage and con-

*Corresponding author. Tel.: +44-121-414-3289; fax: +44-121-414-3263.
E-mail address: L.Seymour@bham.ac.uk (L.W. Seymour)

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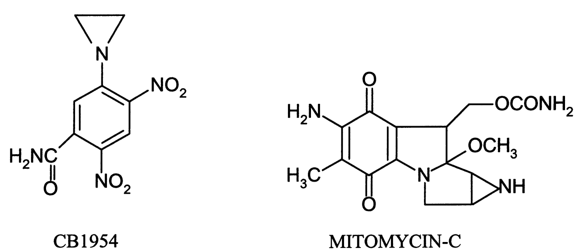

Fig. 1. Chemical structures of CB1954 and Mitomycin C.

sequent apoptotic cell death. The enzyme can also activate the 2-nitro group of CB1954, although the corresponding product does not yield highly cytotoxic species [9].

For effective in situ activation of CB1954, it is important that the prodrug demonstrates pharmacokinetics permitting the drug to be present at target sites at concentrations leading to generation of therapeutic levels of active drug. Hence an understanding of the biological distribution of the drug is fundamental to optimising its use in complex GDEPT protocols.

As a prelude to a forthcoming series of clinical trials, using CB1954 either as a single agent or in combination with adenovirus encoding the nitroreductase gene (*ntr*) to treat ovarian carcinoma, here we report a simple and rapid HPLC assay for determination of CB1954 in samples of human plasma. The method is based on liquid phase extraction of CB1954 from plasma, its concentration, and then reversed-phase chromatographic analysis using spectrophotometric detection. For calibration, serum samples are spiked with Mitomycin C (MMC) as an internal standard (Fig. 1), and the methodology permits routine determination of CB1954 at levels from 5000 ng/ml to 12.5 ng/ml in plasma samples.

2. Experimental

2.1. Chemicals and solvents

MMC was purchased from Sigma (Dorset, UK); CB1954 was obtained from Cobra Therapeutics (CB1954 stock 5.0 mg/ml in ethanol). All solvents and remaining chemicals were AR grade, and purchased from Fisher (Loughborough, UK).

2.2. Samples of human plasma

For calibration studies and determinations of extraction efficiency, citrate phosphate dextrose-treated human plasma was obtained from the Regional Blood Transfusion Centre. Analysis of CB1954 was performed using plasma samples isolated from a cancer patient enrolled on a Phase I trial of CB1954. Drug was administered intravenously in saline at a dose of 6 mg/m^2 by short infusion, and blood samples were collected from the contralateral arm, collected in lithium–heparin tubes and then centrifuged at 1000 *g* for 20 min. The supernatant plasma was then transferred to a polypropylene tube and stored at −30°C until analysis. Prior to analysis samples were thawed at 30°C and then centrifuged at 13 000 *g* to remove any particulate matter before use.

2.3. Preparation of standard curve

The standard curve was prepared by adding 100 μl of a serially diluted CB1954 solution in ethanol to blank plasma (1 ml). The resulting samples contained from 12.5 to 5000 ng/ml CB1954. The internal standard MMC (500 ng in 100 μl water) was added to these samples and also to unknown samples.

2.4. Extraction of CB1954 from plasma and sample concentration

MMC (500 ng in 100 μl water) was added to plasma (1 ml) in a 15 ml polypropylene tube, followed by the addition of phosphate buffer (pH 7.0, 1.0 *M*, 100 μl). Extraction and precipitation was accomplished by the introduction of ethanol (6 ml), followed by vortex mixing for 15 min. After mixing the sample was centrifuged at 3210 *g* for 15 min. The supernatant (one phase) was decanted into a fresh 15 ml polypropylene tube leaving behind the precipitate and dried by vortex evaporation (Rapidvap, GRI, Essex, UK, settings, 40°C, 85% speed, 27 mm·Hg approximately 90 min). Due to latent heat of evaporation, these experimental conditions maintained the sample at less than 10°C throughout the drying procedure. Once dry the sample was resuspended in 200 μl of mobile phase A. The

resulting solution was centrifuged at 13 000 *g* for 15 min, 120 μl was taken into a polypropylene autosampler vial and 80 μl was injected onto the HPLC.

2.5. HPLC system

The separation was achieved using a Kontron system comprising of two 422 pumps, 465 cooled autosampler (10°C) and 440 diode array detector controlled by D450 MT2 DAD data system. The chromatographic column used was a Phasesep Hypersil 5 μm ODS, 250×4.6 mm (Cat. no. psl847373). CB1954 and MMC analytes were detected by UV absorption. The diode array detector was set to scan the absorption from 240 to 600 nm with specific channels set to 260, 340 and 414 nm, and quantification was based on peak area at 340 nm. The wavelength 260 nm is associated with non-specific absorption characteristic of many molecules, while 414 nm coincides with the absorption of the 2-hydroxyethyl derivative of CB1954, one of its major degradation products.

2.6. HPLC running conditions

Mobile phase was formed by mixing the following solutions:

- Buffer A 17.5% acetonitrile: 82.5% 0.02 *M* phosphate buffer pH=7.0
- Buffer B 50% acetonitrile: 50% 0.01 *M* phosphate buffer pH=7.0

The elution was performed using the following gradient conditions:

- 0–15 min 100% A; 15–16 min linear change to 100% B; 16–26 min 100% B; 26–27 min change back to 100% A

3. Results

3.1. Detection of internal standard and CB1954

MMC was evaluated as a suitable internal standard, intended for routine addition to samples of plasma before solvent extraction and concentration. MMC has significant absorption at 340 nm and is easily extracted from plasma by ethanol precipitation of the protein. Like CB1954 it also possesses an aziridinyl group and is stable in aqueous solutions at pH 7. Under the conditions described above, MMC was found to be extracted reproducibly from plasma into the alcoholic phase, and subsequently displayed a single peak on HPLC, with retention time 5.85±0.015 min (n=6). The stability of CB1954 in human plasma was assessed by repeat determinations of drug concentration in samples stored as detailed. No deterioration was observed in plasma levels over a period of 5.5 months when stored at −30°C in the dark (103% of original value after 5.5 months) including one freeze thaw cycle. CB1954 stored in ethanol under the same conditions shows negligible deterioration over 12 months. CB1954 showed efficient extraction (96%, coefficient of variation 2.8%, n=3). This was determined by comparing an unextracted water sample (10 μg/ml) with a plasma sample (10 μg/ml) extracted as described in the Experimental Section, and HPLC analysis produced a single peak at retention time 10.72±0.063 min (n=6). CB1954 has maximal absorption at both 260 and 340n m we chose 340 nm because there is less interference at that wavelength. We routinely check the absorption at 260 to confirm that the peak we are measuring is CB1954. There was no indication of detection of any metabolites of CB1954.

The chromatogram shown in Fig. 2 is from blank plasma obtained from a healthy donor. There are several signals with short elution times, representing hydrophilic components of plasma. Above 4 min, only a few small signals were observed. When compared with plasma that had been spiked with MMC (500 ng/ml) and CB1954 (1000 ng/ml) (Fig. 3) the signals representing the two analytes can be clearly identified and accurately resolved.

3.2. Accuracy and precision

Determination of CB1954 showed a high level of precision and accuracy, as detailed in Table 1. The limit of detection, defined as three-times the background noise, was found to be 2.9 ng/ml, this was calculated by comparison of the peak areas of

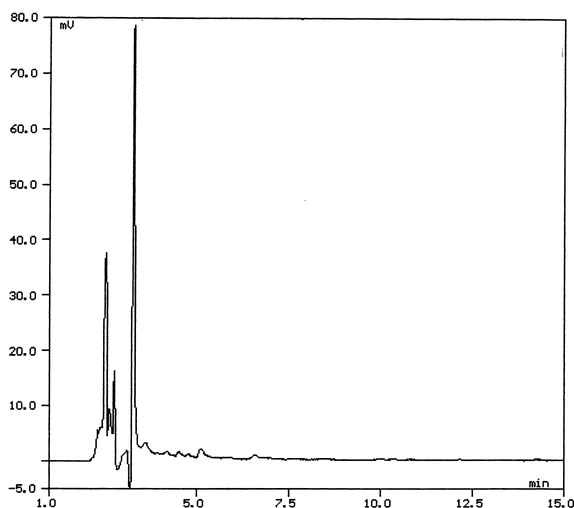

Fig. 2. Chromatographic trace of normal human plasma extracted and analysed as described in Section 2.4.

samples containing 12.5 ng/ml with blank samples over the same time period.

3.3. Analysis of plasma samples from a patient with cancer

Fig. 4 shows the trace generated using plasma taken from a patient at the end of infusion, following

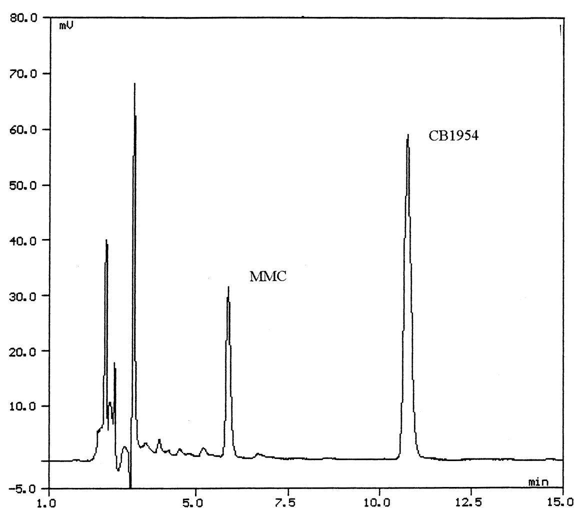

Fig. 3. Chromatographic trace of normal human plasma spiked with Mitomycin C (500 ng/ml) and CB1954 (1000 ng/ml) and extracted and analysed as described in Section 2.4.

intravenous administration of CB1954. The peak relating to the MMC internal standard can be identified clearly, together with that relating to CB1954. One additional small peak can be seen, with retention time 6.54 min. This signal is appears in all samples and is unrelated to CB1954 metabolism.

When results from serial plasma samples taken from this patient are combined (Fig. 5), the profile of CB1954 present in the plasma can be seen to fall rapidly following drug administration. In this case the profile observed fits a biphasic model, with α half-life 14.6 min, and β half-life 170.5 min.

4. Discussion

CB1954 is currently being evaluated for use as a prodrug in preclinical and clinical gene therapy treatment protocols within this Institute. The first stage of the clinical evaluation is a Phase I study of CB1954, aimed at examining the toxicity and efficacy of the drug alone, and also to identify its pharmacokinetics. Subsequently the prodrug will be applied together with the nitroreductase gene, delivered to the tumour using an adenoviral vector, in order to achieve enzymatic activation of the prodrug selectively within tumour cells. To optimise tumour treatment, it is important that the CB1954 prodrug is available to tumour cells at a concentration suitable for its conversion to therapeutic levels of the activated drug. The purpose of the present study is to develop a chromatographic assay system capable of measuring CB1954 accurately within patient samples, and permitting determination of pharmacokinetic parameters for the drug in order to guide its clinical application.

Conventional methodology for measurement of plasma CB1954 employs a direct injection protocol, with plasma samples analysed directly using a strong cation exchange (SCX) column. The detection limit using this system is 600 ng/ml approximately [10]. The analytical system described here includes liquid phase extraction and concentration steps, resulting in a much lower limit of detection (2.9 ng/ml plasma). The signals for CB1954 and MMC are well separated, and can be clearly resolved from any endogenous peaks. In addition, MMC shows very consistent efficiencies of extraction and appears to be a suitable

Table 1
Accuracy and precision of determination of CB1954 from human plasma[a,b]

Actual concentration	Calculated concentration (Mean±S.D)	Coefficient of variation (%)	Accuracy (%)	n
2000 ng/ml	2060±27.9	1.4	103	9
125 ng/ml	136±4.71	3.5	109	9

[a] Linearity of standard curve 5000 to 0 ng/ml=0.9995±0.00005; n=3; 1000 to 0 ng/ml=0.9993±0.00005; n=3; 250 to 0 ng/ml=0.9970±0.00400; n=3.

[b] Limit of detection (defined as three-times background noise) 2.9 ng/ml.

internal standard for this assay. The use of 50% acetonitrile at the end of each chromatographic run is useful as it enhances the elution of some strongly retained materials and thereby accelerates the chromatography.

The concentration of CB1954 required to kill 50% of nitroreductase expressing cell lines in vitro ranges from 0.1 μ*M* (25 ng/ml) for NIH3T3 fibroblasts [4], 2.7–12 μ*M* (680 to 3024 ng/ml) for Skov3 ovarian carcinoma [5] to 9 μ*M* (2268 ng/ml) for Suit2 colorectal carcinoma cells [5]. Hence, although plasma levels of CB1954 cannot be assumed to be the same as levels within tumour cells, the high sensitivity of the assay developed here should permit measurement of plasma CB1954 over a range of concentrations that may lead to therapeutic benefit.

Increased sensitivity of CB1954 detection enables precise analysis of pharmacokinetics, and particularly improves the measurement of area under curve (AUC) values which can be influenced significantly by persistent low levels of the drug in plasma. In clinical studies, particularly those where the patient has received the *ntr* gene, it is possible that a correlation may emerge between anticancer efficacy/toxicity and either maximum plasma levels of CB1954 or plasma AUC. In addition it may be possible to extrapolate levels of CB1954 within tumour tissue, particularly to ascertain whether CB1954 concentration approaches K_M concentrations for *ntr*. Insight into these processes will help to identify factors affecting and limiting generation of activated drug in vivo. Indeed, in later studies, it may be feasible to employ pharmacokinetic assays to predict pharmacodynamic effects, and this raises the possibility of pharmacokinetics-led dose scheduling for individual patients in order to maximise therapeutic benefit.

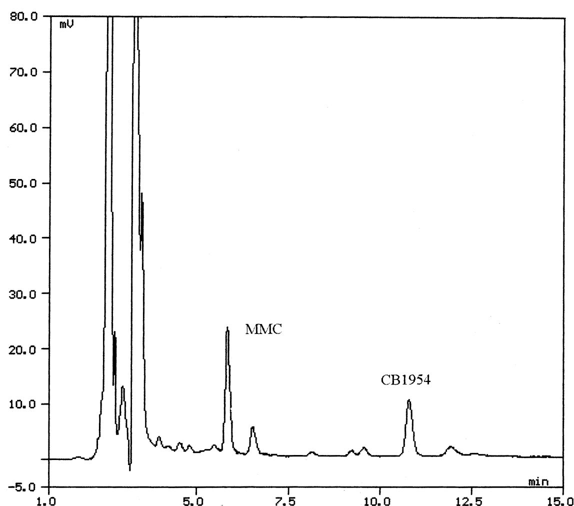

Fig. 4. Chromatographic trace of patient plasma sample isolated at the end of CB1954 infusion (at a dose of 6 mg/m^2) and extracted and analysed as described in Section 2.4.

Analysis of patient samples presented here shows no evidence of any metabolites of CB1954 in plasma samples. Although this patient has not been administered the *ntr* gene, there are endogenous enzymes (such as DT-diaphorase) that are capable of activating the prodrug, albeit with relatively low efficiency [11,12]. We anticipate that the assay system described should be capable of resolving the main metabolites of CB1954 (particularly the 2- and 4-hydroxylamine forms, which are known to elute before CB1954 using the SCX column). It is therefore encouraging that no metabolites have been detected so far, since this is compatible with the possibility that activation of the drug by endogenous enzymes may be ineffective. When the prodrug is used in combination with exogenously-applied *ntr* gene, increased enzymatic conversion may make it possible to detect metabolites of CB1954. On the

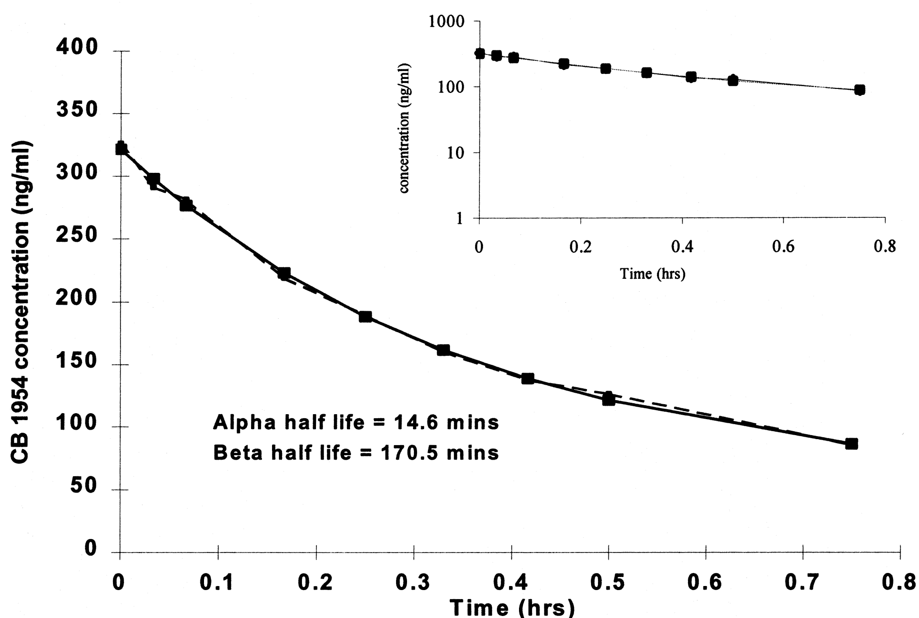

Fig. 5. Blood clearance profile of CB1954 in a patient with cancer, treated intravenously at a dose of 6 mg/m^2. The data (broken line) has been fitted to a biexponential equation (solid line) from which estimates of the AUC and half-lives have been obtained. Plasma was extracted and analysed using the procedure described in Section 2.4.

other hand, such metabolites are extremely reactive and may not ever be released into the bloodstream; indeed, if they do reach the bloodstream they may rapidly alkylate plasma proteins and thereby resist solvent extraction. In many ways it is to be hoped that metabolites will not be detected in the bloodstream, since their presence may well correlate with nonspecific toxicity of the activated drug, leading to unwanted side effects. Hence it is not yet clear whether we shall observe metabolites in future clinical studies, and particularly whether accurate quantification will be possible. Analysis of plasma samples from trials combining CB1954 with *ntr* gene is awaited with interest.

Acknowledgements

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References

[1] T.A. Connors, R.J. Knox, Stem Cells 13 (1995) 501.
[2] T.A. Connors, Gene Ther. 2 (1995) 702.
[3] G.M. Anlezark, R.G. Melton, R.F. Sherwood, B. Coles, F. Friedlos, R.J. Knox, Biochem. Pharmacol. 44 (1992) 2289.
[4] J.A. Bridgewater, C.J. Springer, R.J. Knox, N.P. Minton, N.P. Michael, M.K. Collins, Eur. J. Cancer 31 (a) (1995) 2362.
[5] I.A. McNeish, N.K. Green, M.G. Gilligan, M.J. Ford, V. Mautner, L.S. Young, D.J. Kerr, P.F. Searle, Gene Ther. 5 (1998) 1061.
[6] A.B. Mauger, P.J. Burke, H.H. Somani, F. Friedlos, R.J. Knox, J. Med. Chem. 37 (1994) 3452.
[7] F. Friedlos, S. Court, M. Ford, W.A. Denny, C. Springer, Gene Ther. 5 (1998) 105.
[8] D. Drabek, J. Guy, R. Craig, F. Grosveld, Gene Ther. 4 (1997) 93.
[9] R.J. Knox, F. Friedlos, P.J. Biggs, W.D. Flitter, M. Gaskell, P. Goddard, L. Davies, M. Jarman, Biochem. Pharmacol. 46 (1993) 797.
[10] R.J. Knox, M.P. Boland, F. Friedlos, B. Coles, C. Southan, J.R. Roberts, Biochem. Pharmacol. 37 (1988) 4671.
[11] M. Belinsky, A.K. Jaiswal, Cancer Metastasis Rev. 12 (1993) 103.
[12] R.J. Knox, F. Friedlos, R.F. Sherwood, R.G. Melton, G.M. Anlezark, Biochem. Pharmacol. 44 (1992) 2297.