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Analysis of the imidazoacridinone C1311 by high-performance liquid chromatography

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Abstract

The imidazoacridinone C1311 has shown anti-tumour activity both in vitro and in vivo, prompting its acceptance for Phase I clinical trials. A high-performance liquid chromatography method using fluorescence detection has been developed for the analysis of C1311 in mouse and human plasma and mouse tissue samples. This method is selective, sensitive (limit of detection of 1 ng ml^{-1}) and reproducible, with recoveries of >90%. C1311 was stable over 8 h, at 25°C, in plasma, tumour homogenate, saline and a range of buffers (pH 3.0–8.0). The compound was highly protein bound (>90%) in plasma which may have important consequences in the pharmacokinetics of the drug.

Keywords: Imidazoacridinone; C1311

1. Introduction

The novel 5-[(aminoalkyl)amino)imidazo[4,5,1-*de*]acridin-6-one anti-tumour compounds C1311 and C1310 (Fig. 1) are members of a new rationally designed group of drugs termed the imidazoacridinones. The development of this group of compounds was based on the results of studies on the mechanism of action of mitoxantrone (Fig. 1) which was shown to form interstrand cross links in DNA via the diaminoalkyl groups, the presence of which were found to be a prerequisite for biological activity [1]. Acridinone (and the parent compound acridine) are both known to be classic DNA intercalators which prompted the development of acridinone derivatives bearing a diaminoalkyl side chain. The imidazoacridinones were synthesised on this basis [2].

The major features of the imidazoacridinones are a planar, polycyclic nucleus (capable of DNA intercalation) and one or more ethylenediamine side chains. In addition, an imidazole ring was attached to the aromatic ring structure in order to increase the electron density of the π-system, making the polycyclic nucleus more resistant to enzymatic reduction to radical species [2] (the major problem with many anthraquinone derived compounds). The presence of the hydroxyl group at position 8 of the acridinone moiety appears to be very important in the growth inhibiting activity of these compounds [3].

Several acridine derivatives have previously been evaluated for anti-tumour activity in the past, probably the most studied being nitracrine. The problem with nitracrine was its potent myelotoxicity which led to it being discarded after clinical trials.

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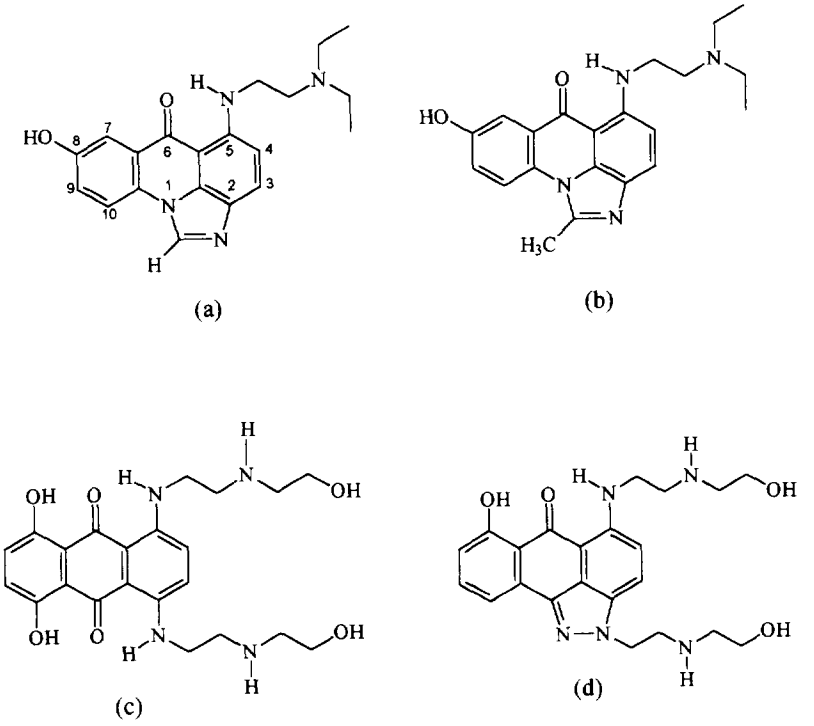

Fig. 1. Chemical structures of (a) C1311, (b) C1310, (c) mitoxantrone and (d) CI-941.

In preclinical in vitro studies, all of the imidazoacridinones showed significant cytotoxic activity against $HeLaS_3$ cells in culture [2] and L1210 leukaemia [4]. In vivo studies have shown significant anti-tumour activity against the murine P388 leukaemia [2]. The imidazoacridinone C1311 has also shown very promising in vivo anti-tumour activity in the models studied in this laboratory [5] which, in conjunction with the absence of observable toxicity at therapeutic doses, has prompted forwarding of the compound for Phase I clinical trials. Therefore a thorough preclinical evaluation of this compound, including pharmacokinetic analysis is of vital importance to its further development in a clinical setting.

The aim of this study was to develop a sensitive and selective method for the quantification of compound in the biological tissues of experimental animals in preclinical evaluation, and to enable pharmacokinetic monitoring in human subjects in a Phase I trial.

High-performance liquid chromatography (HPLC) has been the method of choice for the analysis of related compounds. Several methods have been proposed for the analysis of mitoxantrone using reversed-phase HPLC, with a range of sample clean up procedures including solid-phase extraction and liquid–liquid extraction [6–11]. A reversed-phase HPLC method has been described for the related anthrapyrazole CI-941 (Fig. 1) using solid-phase extraction and detection at 492 and 385 nm [12], but there is no current method available for the analysis of C1311.

In this study, we describe the development of a simple, sensitive and selective reversed-phase method, with fluorescence detection, for the analysis of C1311. The method has been applied to the study of protein binding and stability of C1311 and is suitable for the analysis of compound in biological samples. It will allow plasma and tissue concentrations of C1311 to be determined at levels associated with therapeutic dosing.

2. Experimental

2.1. Chemicals and reagents

Compounds C1311 and C1310 (internal standard) were supplied by Professor J. Konopa (Technical University of Gdansk, Poland). All solvents were HPLC grade. Acetonitrile was purchased from Fisher Scientific (Loughborough, UK). Disodium orthophosphate (I) and citric acid (HPLC grade) were purchased from Merck (Lutterworth, UK). Triethylamine was purchased from Sigma (Poole, UK). Soda glass test tubes, orthophosphoric acid (II) and potassium dihydrogen orthophosphate (III) (analytical grade) were purchased from Fisher Scientific. The buffers for stability analysis were: pH 3.0: 0.1 *M* III–0.1 *M* II and pH 5.0 and 8.0: 0.1 *M* III–0.1 *M* I.

2.2. Instrumentation

UV–Vis spectra were measured on a Beckman DU-650 spectrophotometer (Beckman, High Wycombe, UK) over the range 225–550 nm. The chromatographic system consisted of a Waters 510 pump and Waters 717 autosampler (Waters, Watford, UK). Detection was via a Merck F1050 fluorescence detector using an excitation wavelength of 420 nm and an emission wavelength of 520 nm, and results recorded on a Varian 4290 integrator unit (Varian Assoc. Ltd., Warrington, UK). Diode array detection was performed using a Waters 996 photodiode array detector (Waters).

2.3. Chromatographic conditions

Chromatographic separation was achieved using a Spherisorb S5 ODS-1 (5 μm, 250×4 mm) C_{18} endcapped cartridge (Phase Separations, Deeside, UK) with a LiChrospher 100 RP-18 (5 μm) guard column (Merck).

The mobile phase consisted of acetonitrile–disodium orthophosphate/citrate buffer (50:50, v/v) containing triethylamine (TEA) (0.07% v/v). A 500-μl volume of TEA was added to 250 ml disodium orthophosphate (0.06 *M*) and adjusted to pH 4.0 using citric acid (0.03 *M*). The flow-rate was maintained at 1.5 ml min^{-1}.

2.4. Assay validation

Stock solutions of C1311 and C1310 at 1 mg ml^{-1} were prepared by dissolving appropriate weights of compound in 0.9% saline in soda glass test tubes and stored at −20°C. Standards were then prepared at required concentrations by subsequent dilutions in saline of 1 mg ml^{-1} stock solutions.

C1311 was extracted from mouse plasma or tumour tissue homogenates by addition of acetonitrile. Volumes (100 μl) of samples were obtained and spiked with internal standard (C1310 in saline) to a concentration of 1 μg ml^{-1} plasma or 1 μg g^{-1} tissue. Acetonitrile was then added to give a final ratio of 1:2 (sample–acetonitrile, v/v). Samples were then centrifuged for 15 min at 5000 *g*. Tissues were homogenised 1:3 in mobile phase buffer and analysed as for plasma samples. Concentrations of C1311 were calculated per gram tissue. Supernatants were placed into glass autosampler conical inserts (Jones Chromatography, Hengoed, UK) and injected onto the HPLC system.

Standard samples over the range 0.01–10.0 μg ml^{-1} were produced by spiking mouse plasma and tissue homogenate samples with known quantities of C1311 and internal standard. Calibration curves were obtained by plotting of the peak-area ratios (C1311 peak area/C1310 peak area) against known standard concentrations. Samples containing concentrations of drug above the range of the calibration curve were diluted to within the range of the curve and re-extracted.

C1311 spiked plasma and tissue homogenate samples were prepared ($n=6$) for each of three concentrations (0.1, 1.0 and 10.0 μg ml^{-1}). Extraction efficiencies were calculated by the comparison of extracted standards with non-extracted saline controls and expressed as a percentage of compound recovered.

Between-day variation was performed by preparing calibration curves on six consecutive days and calculating accurate concentrations for 0.1, 0.6 and 1.0 μg ml^{-1} from the calibrations.

2.5. Stability of C1311

Stability was evaluated by incubation of C1311 and C1310 standards in a range of media including mouse plasma, tissue homogenate and buffers at pH 3.0, 5.0 and 8.0, over a period of 8 h at 25°C. Samples were taken at 1-h intervals, extracted as described and peak areas expressed as a percentage of those observed at $t=0$. Half-lives were calculated using log linear regression analysis.

2.6. Protein binding

Initially, protein binding of C1311 to plasma protein was studied using ultrafiltration. Ultrafiltration was performed using Centrifree micropartition devices (Amicon, Gloucester, UK). Murine plasma samples were spiked with C1311 to a range of final concentrations (1, 10, and 100 μg ml^{-1}). Triplicate samples were then subjected to ultrafiltration (5000 *g* for 10 min) and filtrates assayed for C1311 using HPLC.

In addition, relevant filtered saline controls (used to assess the extent of drug binding to filters) and non-filtered saline controls (taken as 100%) were used. Protein binding and filter binding of C1311 were calculated as percentage of the non-filtered saline controls (100%).

Due to the high level of drug binding to the filter devices (as described in results), dialysis was also evaluated. Dialysis tubing (cellulose acetate, 10 mm diameter, purchased from Sigma) was used in a limited study with a 500 μg ml^{-1} C1311 solution in saline.

Further to this, ultracentrifugation was used. Ultracentrifugation was performed using a Beckman Optima TL ultracentrifuge equipped with a Beckman TLA 100.4 rotor (Beckman). Human plasma samples, spiked to a range of final C1311 concentrations (0.1, 1.0, 10, 100 and 625 μg ml^{-1}), were centrifuged for 16 h at 250 000 *g* (77 000 rpm) [13]. Human plasma was used due to the requirement for large volumes in the centrifugation process. Supernatants were analysed directly by HPLC. Plasma samples were compared to identically treated saline controls.

3. Results

3.1. Assay validation

The absorbance spectrum observed following wavelength scan of a 1 μg ml^{-1} solution of C1311 in pH 3.0 buffer over the range 225–550 nm showed regions of maximal absorbance at 250 [*A* (1%, 1 cm)=1050], 272 [*A* (1%, 1 cm)=720] and 420 nm [*A* (1%, 1 cm)=660] (Fig. 2).

Subsequent fluorescence spectrum studies at an excitation wavelength of 420 nm showed 520 nm as a suitable emission wavelength for fluorescence analysis of C1311 and internal standard. The detection limit observed was 1 ng ml^{-1} (signal-to-noise ratio >3:1) equivalent to an on column injection of 0.01 ng.

The addition of the amine modifier TEA to the mobile phase was performed in order to prevent peak tailing. The influence of pH on the separation of C1311 and internal standard was assessed by the use of buffer components at pH 3.0 and pH 5.0. The incorporation of a buffer component at pH 4.0 provided sufficient separation (R_s=2.00) of the compounds with retention times varying by <1%. The use of a more acidic buffer component (pH 3.0) showed deterioration in peak shape with peak broadening observed. An increase in buffer pH (pH 5.0) brought about increases in retention times (from 6.5 min to >30 min in the case of C1311).

The calibration curves produced for C1311 from both plasma and tissue homogenate were linear over the range 0.1–10.0 μg ml^{-1}. Calibrations curves generated for the range 0.1–1.0 μg ml^{-1} were linear

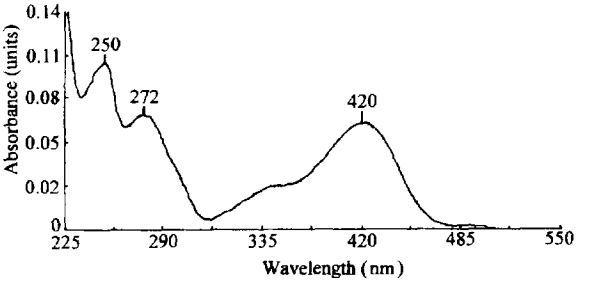

Fig. 2. Absorbance spectrum for C1311 (1.0 μg ml^{-1} in pH 3.0 buffer) over the range 225–550 nm.

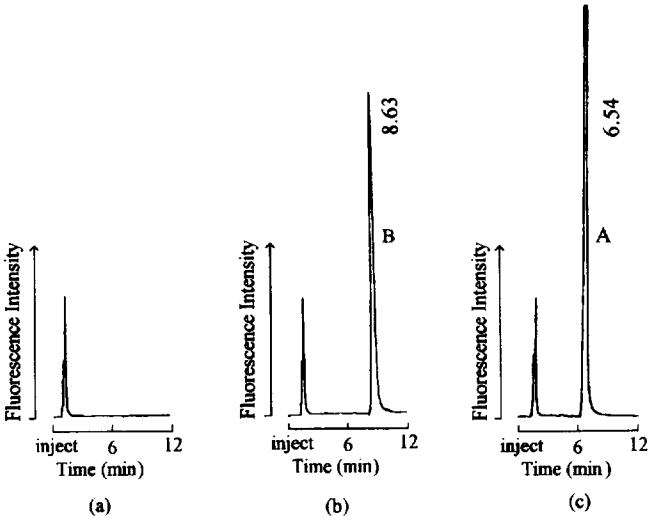

Fig. 3. Examples of typical chromatographic profiles. (a) Blank mouse plasma. (b) Blank mouse plasma spiked with C1310 (1 μg ml^{-1}) (B), (c) Blank mouse plasma spiked with C1311 (1 μg ml^{-1}) (A).

(r>0.997 for both plasma and tissue homogenate) with intercepts not appreciably different from zero and gradients of 2.46±0.27 (mean±1 S.D.). Extraction efficiencies at C1311 concentrations of 0.1, 1.0 and 10.0 μg ml^{-1} were >90% [with coefficients of variation (C.V.)<5%] for both plasma and tissue homogenate.

The C.V. for inter-day variability of the assay of spiked mouse plasma or tissue homogenate on five different days ranged from 9% at 0.1 μg ml^{-1} to 2% at 1.0 μg ml^{-1}. Intra-day C.V.s were always below 5% (n=6).

Comparison of the chromatography for blank mouse plasma (Fig. 3a) and tissue homogenate with internal standard (Fig. 3b) and C1311 (Fig. 3c) spiked samples showed the absence of interference from endogenous co-extracted compounds as did analysis of blank extracted human plasma samples.

Subsequent analysis of extracted plasma and tissue samples derived from C1311-treated NMRI (Naval Medical Research Institute) mice [injected at 50 mg kg^{-1} intraperitoneally (i.p.)] showed the suitability of the extraction and analytical method in the identification and quantification of C1311 concentrations from in vivo experiments (Fig. 4). The identity and purity of C1311 extracted from both mouse plasma and tissue (liver) was confirmed using photodiode array detection (at concentrations >200 ng ml^{-1}).

3.2. Stability of C1311

Analysis of C1311 (1 μg ml^{-1}) in mouse plasma

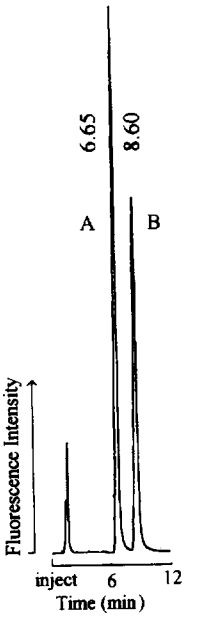

Fig. 4. Example of a typical chromatographic profile of a plasma sample from a C1311-treated NMRI mouse (30 min after a dose of 50 mg kg^{-1} i.p.). (A) C1311, calculated as 0.63 μg ml^{-1} from calibration. (B) C1310 (internal standard), 1 μg ml^{-1}.

at 25°C showed no appreciable decomposition as assessed by the slope of the concentration versus time plot. The study was performed over a period of 8 h (25°C) with an additional study following incubation over a period of 24 h. In addition, C1311 was stable (with calculated half-lives of >100 h) in a range of buffers (pH 3.0, 5.0, and 8.0) as well as tissue homogenate and 0.9% saline which was used as the diluent of choice for the compounds.

3.3. Protein binding

Initial work in assessment of a method for the evaluation of the extent of plasma protein binding of C1311 involved the use of a range of centrifugal filters. However analysis of C1311 spiked mouse plasma samples in comparison to filtered and non-filtered saline controls showed a high degree of binding to the filters themselves. Cellulose acetate filters were assessed with filter bound drug consisting of approximately 85%, 89% and 19% of the total applied drug for filtered saline controls at 1.0, 10.0, and 100.0 μg ml^{-1} respectively. Taking the filter bound drug into account allowed calculation of actual protein bound drug (Table 1).

In addition, preliminary evaluation of dialysis for the assessment of C1311 plasma protein binding (using saline) showed very slow approach to equilibrium with <5% of applied drug having diffused out of the tubing after 24 h.

Ultracentrifugation of C1311 spiked plasma samples proved to be the only alternative method which could provide a reliable answer to the protein binding analysis. The comparison of triplicate ultracentrifuged human plasma samples with non-centrifuged saline controls enabled estimation of the percentage of binding occurring between the compound and plasma proteins. Comparison of these samples showed protein binding to be approximately 99%, 99%, 99%, 85% and 60% for 0.1, 1.0, 10.0, 100.0 and 625.0 μg ml^{-1} respectively (Table 2). Analysis of saline controls at each concentration showed that centrifugation had no effect on C1311 concentrations.

Table 1
Extent of mouse plasma protein and filter binding of C1311 ($n=3$, mean±1 S.D.)

C1311 concentration (μg ml^{-1} plasma)	Filter bound (%)	Protein bound (%)
1.0	85.5± 0.9	87.5±16.0
10.0	88.7± 6.8	72.4±21.1
100.0	19.2±30.1	94.9±47.1

Table 2
Extent of binding of C1311 to human plasma proteins following evaluation of ultracentrifugation as a method for determining plasma protein binding (mean±S.D.)

C1311 concentration (μg ml^{-1} plasma)	Protein bound (% of control)
0.1	98.8±4.1
1.0	99.2±1.4
10.0	98.8±2.2
100.0	85.2±1.1
625.0	59.6±6.3

4. Discussion

The method described takes advantage of the fluorescent nature of the compounds and is highly sensitive for the analysis of compound extracted from both plasma and tissue homogenate with a limit of detection of 1.0 ng ml^{-1} (equivalent to 0.01 ng on column injection). The method is highly selective, with no interference from endogenous co-extracted compounds observed, and reproducible with coefficients of variation consistently <10% and generally <5%. The use of protein precipitation (using acetonitrile) of biological samples with high observed recoveries (>90%) has aided in the simplicity of the procedure. Indeed, sensitivity could be potentially enhanced by the addition of a concentrating step following extraction. The subsequent analysis of extracted mouse plasma and tissue samples using diode array detection allowed the purity of the C1311 peak to be assessed. This, together with peak comparison with a C1311 standard, confirmed the identity of the peak as C1311.

The developed method enabled study of the stability of C1311 in both plasma and tissue homogenate as well as in a variety of buffers and 0.9% saline. C1311 and the internal standard C1310 are stable in both plasma and tissue homogenate for at least 8 h at 25°C which is important for the storage

and handling of the compound standards and tissue samples.

Previous studies on the related compound mitoxantrone showed it to bind readily to the glassware used in sample preparation [10], but this problem was not observed in our work on C1311. Preliminary analysis of plasma protein binding of C1311 involved the use of centrifugal filtration of spiked plasma samples, saline controls and non-filtered saline controls. This study indicated a large degree of non-specific binding to the cellulose acetate filters themselves and prompted the evaluation of further filter types (e.g. polysulfone). Dialysis was also shown to be unsuitable for such studies.

Ultimately, ultracentrifugation was the method used to elucidate the compounds protein binding characteristics. Evaluation of ultracentrifugation provided reliable information with regard to plasma protein binding. Analysis of the percentage of applied compound found to be protein bound (using escalating concentrations) showed C1311 to be highly protein bound (99%) up to applied concentrations of 100.0 and 625.0 $\mu g\ ml^{-1}$ where the initiation of saturation of protein was observed (85 and 60% bound respectively). This high level of protein binding will significantly influence the pharmacokinetics of the compound in humans.

In summary, the potential anti-tumour activity of C1311 has prompted its further development into Phase I clinical trials. We have described a chromatographic method for the analysis of the compound. The method is sensitive, selective and reproducible and will allow the pharmacokinetic study of C1311 both in experimental systems and in a clinical setting.

Acknowledgments

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