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Short communication

Validation of a method for the determination of the anticancer agent Combretastatin A1 phosphate (CA1P, OXi4503) in human plasma by HPLC with post-column photolysis and fluorescence detection

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ABSTRACT

A validated method for the determination of Combretastatin A1 phosphate (CA1P, OXi4503), a bisphosphate prodrug of the vascular disrupting agent Combretastatin A1 in human plasma has been developed using fluorescence detection after post-column photolysis. The separation used the ion-pairing agent tetrabutylammonium hydrogen sulphate, and this agent was also required to give consistently high recovery from plasma. Initially, the range was shown to be linear ($r^2 > 0.995$) from the LOQ of 0.025 μM to 5 μM, but as the trial progressed to much higher doses, using a lower injection volume, the assay was subsequently subject to limited revalidation to cover the range from 0.05 to 50 μM. Intra-assay precision and accuracy ranged from 2.2 to 11.8% and 1.8 to 13% respectively, and for inter-assay from 4.4 to 14.9% and 1.7 to 6.5%. Mean recovery of OXi4503 from plasma was 80.2%.

1. Introduction

Combretastatin A1 phosphate (CA1P, OXi4503, Fig. 1) is a bisphosphate pro-drug of the colchicine analogue Combretastatin A1 (CA1, OXi4500) which acts as a vascular disrupting agent (VDA) by depolymerising tubulin in the endothelial cell, resulting in breakdown of tumour blood flow [1]. It is a more active analogue of Combretastatin A4 phosphate (CA4P) [2,3] and has recently completed a CRUK-supported phase I trial as an anti-cancer agent.

CA1P is a pro-drug activated by dephosphorylation, so ideally an analytical method would also be able to measure the active drug, CA1, as well as the two intermediate monophosphates, and possibly secondary metabolites such as glucuronides, as was the case with CA4P [4]. One method of choice for pharmacokinetic studies in clinical trials is LC–MS. This generally gives higher sensitivity and specificity than UV detection, and not all drugs are directly amenable to more sensitive fluorescence or electrochemical detection. In addition LC–MS has the potential to allow for the detection and identification of unknown metabolites. We therefore expended considerable effort in attempting to develop a separation which resolved all these analytes. However, the constraints on the eluents available for use in LC–MS, and the differing requirements of the pro-drug and the active agent, together with incompatibility of the extraction technique required to get reproducible recoveries of CA1P with LC–MS led us to abandon attempts to determine CA1P and CA1 simultaneously, or to use this detection mode for CA1P determination in plasma. It is notable that the only paper on comparative pre-clinical pharmacokinetics of CA1P and CA4P using MS detection [5] does not show any data on plasma CA1P. We did develop a successful validated method for plasma CA1 by LC–MS which was driven in part by the need to measure the low concentrations of this metabolite seen in the clinical samples, and also by our observation that CA1, unlike the other combretastatins we had studied, was not amenable to photolytically enhanced fluorescence detection [6]. Although CA1P is not naturally very fluorescent, the *trans* isomer is much more so, and this paper describes a validated method for the determination of CA1P in human plasma by HPLC, using post-column photolysis to induce isomerisation, with fluorescence detection.

2. Materials and methods

2.1. Chemicals

Structures of the compounds studied are shown in Fig. 1. CA1P (dipotassium salt) (99.2% pure) was from Evotec (Abingdon, UK), and CA4 (99.3%) used as internal standard (IS) was from Pharm-Eco Laboratories (Devens, Mass., USA). Tetra-n-butylammonium

Abbreviations: CA1(P), Combretastatin A1 (phosphate); CA4(P), Combretastatin A4 (phosphate); IS, internal standard; TBA, tetrabutylammonium; VDA, vascular disrupting agent.

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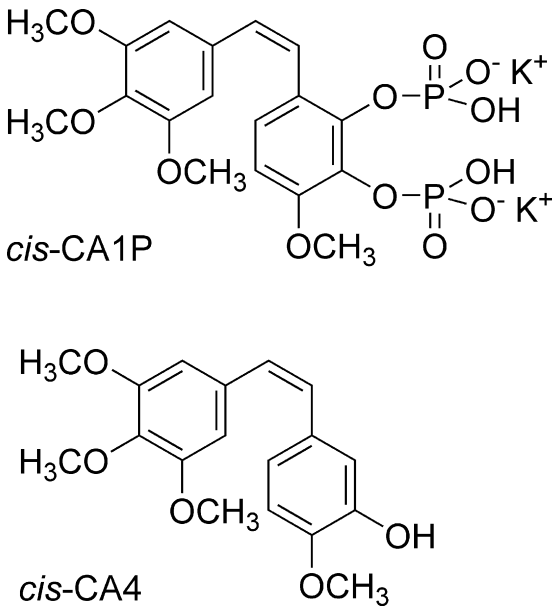

Fig. 1. Structures of the compounds studied.

hydrogen sulphate ($TBAHSO_4$, ECD grade), acetonitrile (far UV grade), and methanol were from Fisher (Loughborough, UK), ascorbic acid and dimethyl sulphoxide (DMSO) were from Sigma (Poole, UK) and dipotassium hydrogen orthophosphate (K_2HPO_4) was from VWR, Lutterworth, UK.

2.2. Standard solutions

Working in reduced lighting, stock solutions of CA1P were prepared at 1 mM in water and diluted appropriately into human EDTA plasma containing 1 mM ascorbic acid. CA4 (IS) was made up as a 1 mM solution in DMSO, diluted to 50 μM in 30% DMSO and stored at −20 °C. On a weekly basis, the 50 μM stock was diluted to a working concentration of 2.5 μM in 30% methanol/water.

2.3. HPLC

The HPLC system consisted of a 2695 separations module and 474 fluorescence detector fitted with a 5 μL flow cell, both from Waters (Watford, UK). During chromatographic development, a Waters 2996 diode array detector was also used to aid peak identification. Chromatographic separation took place on an ACE 3 μm C18 column (150 mm × 3 mm) (Hichrom, Reading, UK) maintained at 35 °C. Solvent A was 20% methanol, 8 mM K_2HPO_4, 5 mM $TBAHSO_4$, solvent B was 75% methanol/water and C, 75% acetonitrile/water. Initial conditions were 73% A, 27% B, with a linear gradient to 45% A, 55% B over 9 min, then to 5% A, 55% B, 40% C over 5 min, and finally to 100% C over 0.5 min, held for 2 min before returning to initial conditions. The flow rate was 0.5 mL/min and the run time was 25 min. Between the column outlet and the detector, a photolysis coil was placed comprising of 75 cm 0.006″ PTFE tubing wound round a mercury lamp from a Waters 441 detector [6]. The fluorimeter wavelengths used were excitation 320 nm, emission 390 nm. Data were acquired using Waters Empower software.

2.4. Sample preparation

All extraction procedures were carried out in reduced lighting. To 50 μL aliquots of plasma, 20 μL IS, 40 μL 0.1 M $TBAHSO_4$, and 1 mL methanol were added, with vortex mixing after each addition. Samples were then kept at 4 °C for 15 min, then spun at 20,000 × *g* for 10 min at 10 °C. The supernatant was decanted into 4 mL amber glass vials and taken to dryness in a heated centrifugal evaporator. The extracts were redissolved by the addition, with mixing, of 40 μL methanol, followed by 60 μL water, and transferred to amber HPLC injection vials containing 200 μL glass inserts. Initially, the injection volume was 40 μL, subsequently reduced to 25 μL for the higher concentration range (0.05–50 μM).

3. Results and discussion

3.1. Chromatographic conditions and separation of CA1P from potential metabolites

In our previous studies with the monophosphate CA4P, we separated the prodrug from the active CA4 using the ion-pairing agent $TBAHSO_4$ at a relatively low pH, which enabled us to quantify both components (and the glucuronide) using either UV absorbance, or for more sensitivity, fluorescence detection [4]. Retention was relatively insensitive to the $TBAHSO_4$ concentration (up to 15 mM), even with higher pH eluents when the ionisation of the phosphate group would be increased, and hence there would be greater scope for ion-pairing to occur, perhaps because of the hydrophobic nature of the stilbene ring system. However, with the bisphosphate CA1P, we found that the peak shape at low pH was rather poor. This effect was investigated further using a fixed concentration of TBA (5 mM), and varying the pH and phosphate concentration. Peak efficiencies were calculated using Empower software. At pH 6.91, with 7 mM phosphate, CA1P showed over 5000 plates; reducing the phosphate to 3 mM using the alkaline TBAOH in place of $TBAHSO_4$ showed reduced efficiency, but still with good peak symmetry. In contrast, at pH 3.13 with the same 3 mM phosphate concentration, the efficiency dropped, and the peak developed severe 'fronting'. The latter could be minimised using 7.5 mM phosphate at pH 3.05, which still showed reduced efficiency, but with a much more symmetrical peak shape. In the absence of any ion pairing agent, with 10 mM phosphate (pH 7.2), as expected, retention was much reduced, but the peak efficiency was also lower (2300 plates). On silica-based composite packings (Gemini and Gemini NX C18, Phenomenex) we observed that the trend to improved chromatography for this drug was continued up to pH 10.2 using ammonium carbonate-based eluents (data not shown). Indeed we have more recently exploited this behaviour in developing a validated method for CA1P in urine at pH 9 using mass spectrometric detection, where the problems discussed below with regard to extraction from plasma are not an issue. We have been unable to find any examples in the literature, with the *ortho*-substituted aromatic structure of CA1P, of the marked deterioration in the peak shape as the pH decreased.

As CA1P is a pro-drug, it was essential that the active metabolite CA1 did not interfere in the analysis. However, in addition, it was known from pre-clinical studies and by analogy with CA4P, that the two possible intermediate monophosphates would be present; glucuronidation was also likely to be an important metabolic route, with two potential monoglucuronides and a diglucuronide. Therefore, bearing in mind the beneficial chromatographic effects noted above of carrying out the analysis using ion pairing at relatively high pH, a method was devised which resolved these components with $TBAHSO_4$, adjusting the pH with K_2HPO_4. In order to facilitate distinguishing the *cis* and *trans* isomers which are present as impurities in the standards to varying degrees, this initial work was carried out using absorbance detection as the two isomers have very different spectra [6]. From our previous experience with CA4P, it was not thought likely that significant amounts of the *trans* isomers would be formed *in vivo*, and this has been our subsequent experience with CA1P. Fig. 2A shows the separation of the parent CA1P from the intermediate monophosphates, the active CA1, and

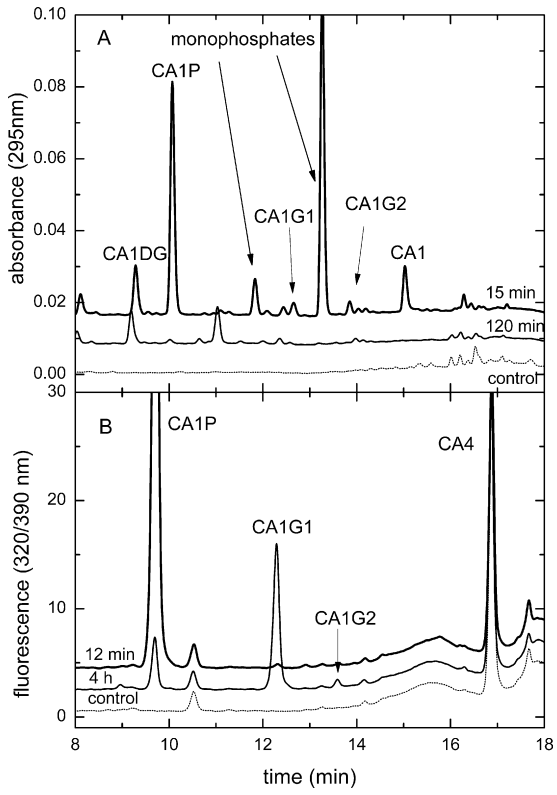

Fig. 2. A: Chromatograms of mouse plasma extracts after CA1P administration (50 mg/kg i.p.) with the same chromatographic conditions as used for the human studies, but with absorbance detection, showing separation from possible metabolites. CA1G1, CA1G2 and CA1DG are the two possible monoglucuronides and the diglucuronide respectively. B: Chromatograms of patient plasma extracted before, and 12 min and 4 h after an i.v. infusion of 14 mg/m^2 CA1P.

three glucuronide metabolites in the plasma from a mouse dosed i.p. with 50 mg/kg CA1P. The identities were assigned from both the retention times, and also the absorbance spectra, which although all showed a peak around 295 nm, had slightly different characteristics. The *trans*-isomer of CA1P, which is not formed in significant amounts *in vivo*, elutes about 0.8 min after *cis*-CA1P. Fig. 2B shows chromatograms of patient plasma extracted before, and 2 min and 4 h after an i.v. infusion of 14 mg/m^2 CA1P. Although the two glucuronides were resolved in this system, slightly better sensitivity was seen at low pH [6], and these metabolites were quantified by repeating the analysis in a phosphate eluent at pH 2.7 (data not shown).

3.2. Extraction of OXi4503 from plasma

Recovery of CA1P from human plasma was determined by comparing the peak areas with those of equivalent aqueous solutions. A number of approaches were assessed to extract the drug efficiently from plasma, including simple methanol or acetonitrile protein precipitation, solid phase extraction (both reversed phase and ion-exchange) with a variety of diluents, and liquid–liquid extraction. Because recovery of CA1P from plasma was so low and variable, formal recovery studies were not performed. For example, solid phase extraction on a polymeric reversed phase column (Strata X) of plasma spiked with 0.2–5 μM CA1P and acidified with 10 mM HCl gave mean recoveries of only 17.5%. This increased to 67% in the presence of 5 mM $TBAHSO_4$ in 20% methanol, and similar recoveries were obtained with liquid–liquid extraction using ethyl acetate to extract acidified plasma salted out with brine. The iron chelator deferroxamine also improved recovery to a small extent, but only

Table 1
Recovery of CA1P from human plasma.

Concentration (μM)	Recovery (%)	%RSD
4.00	78.2	2.9
0.400	76.9	1.9
0.100	81.1	2.6
0.025	84.5	14.7

by incorporating relatively high concentrations of $TBAHSO_4$ (40 μL 0.1 M added to 50 μL plasma) with the plasma could reliable extraction be obtained using methanol precipitation (Table 1), although even with this, the recovery was significantly below 100%. Lower concentrations of $TBAHSO_4$ gave much more variable recoveries which depended on the source of the control plasma. $TBAHSO_4$ is well known to cause ion suppression in MS and the high concentration required for extraction was one of the reasons for rejecting this as a detection technique. We were unable to achieve satisfactory reproducibility using these samples in eluents compatible with mass spectrometric detection, although we have not investigated alternative ion pairing agents which are reported to be suitable for use in MS systems [7]. Notably, this requirement for the ion pairing agent to enhance extraction was species and even strain specific, with some mouse strains giving high recovery using just methanol as extractant. This may relate to the high degree of protein binding seen in man (>99%) relative to the mouse (64%), and is consistent with the data of Kirwan et al. [5] which quotes 91% recovery of CA1P from mouse plasma using acetonitrile as extractant. Although the use of TBA to extract acidic species into organic solvents or enhance retention during solid phase extraction is well documented [8,9], examples of its use to facilitate consistent extraction using simple methanol protein precipitation are not known.

3.3. Specificity

Twelve samples of control human EDTA plasma from different sources were extracted as described above, but without the addition of internal standard, and checked for interferences with either CA1P or the IS. No samples showed any significant co-eluting peaks. Fig. 3 shows a typical chromatogram of a control extract and one spiked with 0.025 μM CA1P and 0.5 μM IS (CA4).

3.4. Linearity

Four calibration curves were obtained by plotting the ratio of the CA1P peak area to the IS area, fitted by linear regression weighted to 1/conc^2. Curves run over the range of 0.025–5 μM were linear with a mean slope of 0.285, %RSD of 9.5, and mean correlation coeffi-

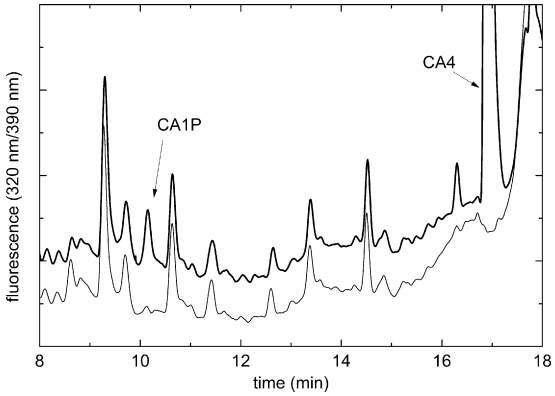

Fig. 3. Chromatograms of a control human plasma extract and one spiked with 0.025 μM CA1P and 0.5 μM IS (CA4).

Table 2
Intra- and inter-assay precision and accuracy.

	Nominal concentration (μM)	Calculated mean concentration (μM)	Precision (%RSD)	Accuracy (%RE)
Intra-assay	40	40.4	2.20	1.00
	4.00	4.07	2.75	1.81
	0.400	0.388	2.17	-3.06
	0.100	0.106	3.14	5.50
	0.025	0.0283	11.7	13.0
Inter-assay	40	43.2	5.46	7.95
	4.00	4.26	6.41	6.47
	0.400	0.407	7.12	1.72
	0.100	0.103	4.37	4.37
	0.025	0.0257	14.9	2.90

$n = 4$ for intra-day, $n = 16$ for inter-day, except for 40 μM, where $n = 2$ and 6 respectively.

cient (r^2) of 0.998 ($n = 4$). Similar linear curves were seen using the extended curve (0.05–50 μM).

3.5. Precision and accuracy

Intra- and inter-day precision and accuracy (%RSD and %RE) were calculated from replicate analyses of QC samples spiked with four different concentrations of CA1P on four separate occasions (Table 2). At 0.025 μM, the intra-assay precision and accuracy were still < 15%, but both increased markedly relative to the higher concentrations and this was taken as the LLOQ.

3.6. Stability

CA1P stability was assessed in whole blood at two concentrations for up to 2 h, and in plasma at three concentrations at −80 °C for 12 months, at −20 °C for 24 h (required because trial samples were stored for up to 24 h at this temperature), at 18–22 °C for 4 h, and after 3 freeze (−80 °C)/thaw cycles. All measurements were within ± 15% and therefore indicated that CA1P was stable under these conditions.

4. Conclusions

This analytical method has been successfully applied to the analysis of ∼450 plasma samples from over 40 patients in a clinical trial of CA1P. No significant interferences were seen in any samples, and the highest concentration of CA1P which had been observed when the trial ended was 16.8 μM, well within the validated range.

Acknowledgement

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