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Determination of combretastatin A-4 and its phosphate ester pro-drug in plasma by high-performance liquid chromatography

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Abstract

High-performance liquid chromatography with both absorbance and fluorescence detection has been applied to the determination of the potential anti-tumour agent combretastatin A-4 and its phosphate ester in murine and human plasma. The presence of different interfering peaks in the two species makes absorbance detection at 295 nm the method of choice for the mouse, and fluorescence detection (295 nm/390 nm) for human plasma. The calibration was linear over the range studied (0.01–50 μM for combretastatin A-4, 0.02–200 μM for combretastatin A-4 phosphate), with quantitation limits of 0.05 μM for both drugs in the mouse, and 0.05 μM and 0.0125 μM for the phosphate ester and free drug, respectively, in human plasma. The method should be useful for pharmacokinetic studies in the forthcoming Phase I clinical trial of combretastatin A-4 phosphate. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Combretastatin A-4

1. Introduction

Combretastatin A-4, a compound isolated from the African tree *Combretum caffrum*, shows structural similarities to the tubulin binding agent colchicine, and has a higher affinity for the colchicine binding site on tubulin than colchicine itself [1]. There is interest in these compounds as anti-cancer agents since they are able to induce irreversible vascular shutdown in tumours [2,3]. Combretastatin A-4 phosphate, a water-soluble ester of combretastatin A-4, shows a broad therapeutic window in experimental systems [4,5], and has been selected for Phase I trials in man. A method has been previously described for the determination of the parent compound by HPLC for drug binding studies [6], but no methods have been published for its measurement in vivo. This paper describes a method for the determination of both agents, and the separation of some of their metabolites in murine and human plasma.

2. Experimental

2.1. Chemicals

Potassium dihydrogen phosphate (KH_2PO_4) (HiPerSolv grade) and glycine were from Merck (Poole, UK) and tetrabutyl ammonium hydrogen sulphate ($TBAHSO_4$) (Electrochemical grade) was from Fisher (Loughborough, UK). Alkaline phosphatase (Bovine Type IS) was from Sigma (Poole, UK).

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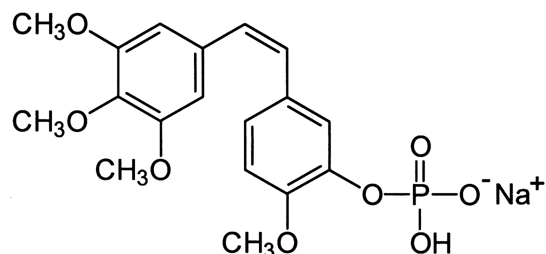
Methanol and acetonitrile were from Rathburns (Walkerburn, UK). *Cis*-combretastatin A-4 and *trans*-combretastatin A-4 phosphate were from Prof. G.R. Pettit, Arizona State University, *cis*-combretastatin A-4 phosphate was from Oxigene Europe AB (Lund, Sweden), and the internal standard (*trans*-3,4,5-trimethoxy-4'-methylstilbene) was a generous gift from Dr. A. McGown, Paterson Institute for Cancer Research, Manchester, UK. The structures of the compounds of interest are shown in Fig. 1.

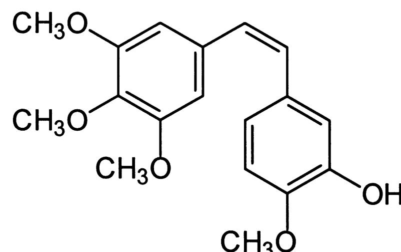
2.2. Samples

Blood samples were collected in EDTA to inhibit endogenous phosphatase activity [7], and plasma collected by centrifugation (12 000*g*, 1 min). To 50 μl of plasma was added 50 μl of internal standard, either 1250 pmol (mouse) or 250 pmol (human) (25 or 5 μ*M* stock in 30% methanol respectively), followed by 500 μl of methanol, with mixing after each addition. Samples were centrifuged to remove precipitated protein (12 000 *g*, 1 min), and the supernatant taken to dryness in amber vials in a centrifugal evaporator (Heto Lab Equipment, Camberley, UK). Samples were reconstituted in 200 μl of 50% methanol/water and transferred to vials ready for analysis by HPLC.

2.3. Chromatography

HPLC was performed using a Waters 616 pump, 717 autosampler, and detection used a Waters 996 diode array detector and 474 fluorescence detector fitted with a 5-μl flow cell. Data was collected and analysed using the Millennium Chromatography Manager (Waters, Watford, UK). Separation was achieved using a base-deactivated RPB column (5-μm particle size) (Hichrom, Reading UK), 250×3.2 mm, with a guard column of the same material, 10×3.2 mm. The column was maintained at 30°C using a column oven (Jones Chromatography, Hengoed, Wales). Eluents comprised A: 15 m*M* $TBAHSO_4$, 3 m*M* KH_2PO_4, pH 2.04; B: methanol; C: acetonitrile. Starting conditions were 50 % A, 50 % B, with a linear gradient over 14 min to 50 % B, 50 % C, returning to the start conditions from 14–15 min, with a flow-rate of 0.6 ml min^{-1}. Preliminary studies were also performed using a Waters Symmetry Shield column (150×3.9 mm), eluents: A: 5 m*M* KH_2PO_4, 5 m*M* H_3PO_4; B: 75 % acetonitrile, 25 % water; gradient 40–85 % B in 5 min, and using a Hypersil APS (amino) column, 250×4.6 mm (Hichrom), eluent 34 % acetonitrile, 20 m*M* citrate phosphate, pH 3.5.





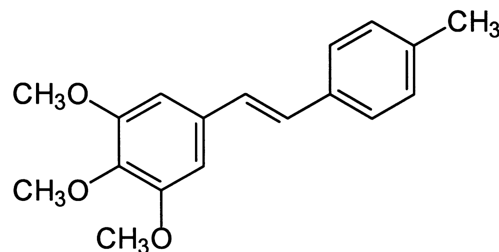


Fig. 1. Structures of the compounds studied.

3. Results and discussion

One of the requirements for the analysis was the separation of the *cis*- and *trans*-isomers of both the

pro-drug phosphate and the combretastatin A-4. Only the *cis*-form is therapeutically active, but conversion to the *trans*-isomer occurs very readily, for example by exposure to light, and may be produced in vivo. Precautions, including the use of amber vials, were taken to prevent exposure of samples during extraction both to direct sunlight and fluorescent strip-lights. We found that the two isomers of combretastatin A-4 were readily separated on most reversed-phase columns, but the phosphate esters were more difficult to resolve. The exception to this was shown by the Symmetry Shield column, which resolved the pro-drug isomers with rather low efficiency, but failed to separate those of the parent (Fig. 2). The phosphate isomers could also be readily separated by ion-exchange using an amino column but again the parent isomers could not (data not shown). The poor efficiency shown by the latter column made it unlikely that a satisfactory separation could be achieved in biological fluids, so further developmental effort was restricted to reversed-phase columns.

A number of these column types were assessed before selecting the base-deactivated Hichrom RPB which, in combination with the ion-pairing agent $TBAHSO_4$, appeared to offer the best peak shape and resolution between the two pro-drug isomers (Fig 3). Methanol was required in the starting eluent to give the separation of these isomers, but the use of an acetonitrile gradient gave superior resolution of peaks present in plasma, without significantly affecting the separation of the isomers. $TBAHSO_4$ had a relatively small effect on the retention of the phosphate esters, but its inclusion gave markedly superior chromatographic peak shapes, and also allowed for the manipulation of the retention of the pro-drug relative to the metabolites of combretastatin A-4. The latter was also aided by the potassium phosphate. The back pressure on the column at the start of each analysis was ~3100 p.s.i., decreasing to $<$2000 p.s.i. at the end of the gradient, and the guard column was changed regularly, after ~150 samples. A dummy gradient was always run before analysis in order to equilibrate the system.

Cis-combretastatin A-4 exhibits lower fluorescence intensity compared to the *trans*-isomer, but, in pure solutions, fluorescence does offer higher sensitivity than absorbance detection. However, we found that in the mouse, a number of interfering

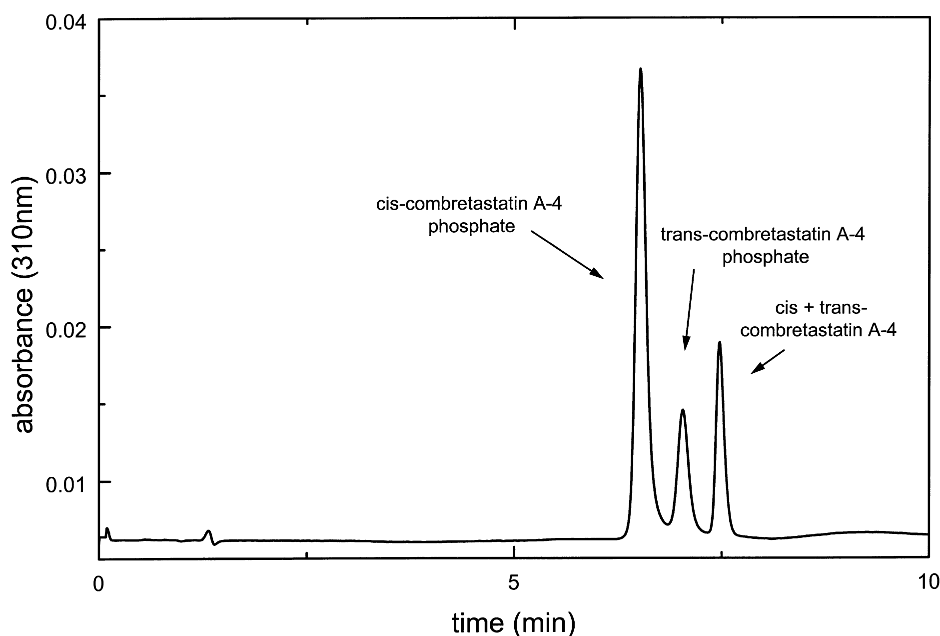


Fig. 2. Separation of the *cis*- and *trans*-isomers of combretastatin A-4 phosphate using a Waters Symmetry Shield column. For chromatographic conditions see Section 2.3.

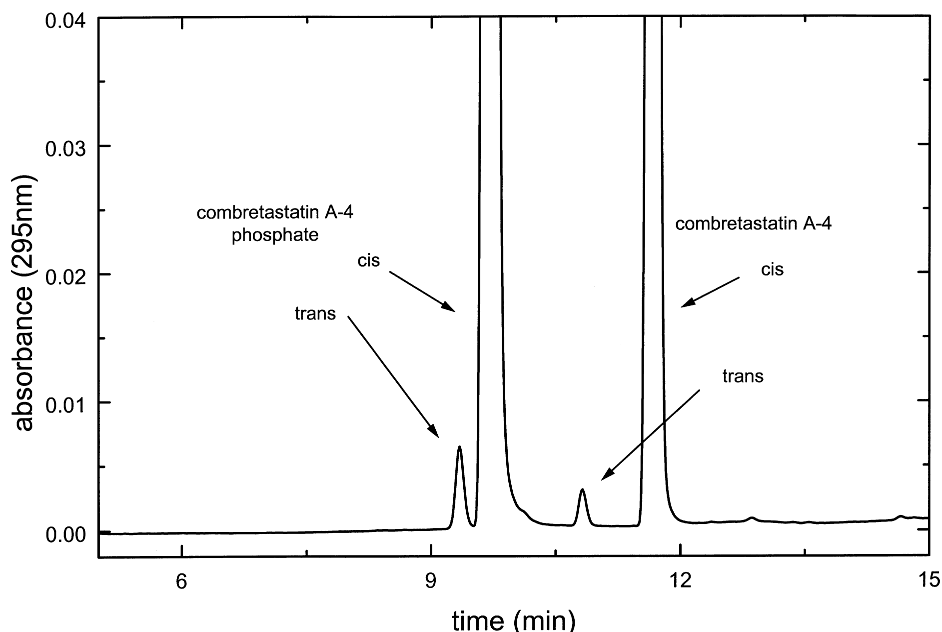


Fig. 3. Separation of the *cis*- and *trans*-isomers of combretastatin A-4 phosphate and the parent combretastatin A-4 using a Hichrom RPB base-deactivated column. For chromatographic conditions see Section 2.3.

fluorescent peaks were extracted, making quantification difficult at low concentrations. Fig. 4a and b show a mouse plasma standard corresponding to 1.4 μM combretastatin A-4 phosphate and 0.35 μM combretastatin A-4 using fluorescence and absorbance detection respectively. In panel a, there is a peak from plasma constituents which co-elutes with the combretastatin A-4 phosphate, whereas in panel b (295 nm) the small peak which elutes ~0.2 min later than the combretastatin A-4 phosphate is readily separated from small drug peaks, permitting good quantitation of >0.1 μM drug. Combretastatin A-4 could be determined using either detection method. In this figure, a large blank peak at ~12.4 min is seen, particularly using absorbance detection. This peak elutes very close to combretastatin A-4 if the low concentration of KH_2PO_4 is omitted from the eluent, even though the phosphate only changes the pH by <0.05 units.

By contrast with the mouse, human plasma has very few interferences using fluorescence detection (Fig. 5a), allowing accurate quantification with this detection mode. With absorbance detection (Fig. 5b) however, blank peaks adversely affect both drug peaks. Use of just a single (fluorescence) detector rather than the two detectors in series should improve the peak shape achieved with the mid-bore (3.2 mm) column employed in this analysis. Both detectors were employed for the development of the assay in order to characterise the spectra of the peaks using the diode array facility. The use of the two different detection modes for mouse and human plasma necessitates the addition of a smaller amount of internal standard where fluorescence detection is used since the internal standard is a *trans*-isomer which is much more fluorescent than the *cis*-form.

Calibration curves were constructed from 0.01 to 50 μM for combretastatin A-4 and 0.02 to 200 μM for combretastatin A-4 phosphate, using absorbance detection at 295 nm for the mouse, and fluorescence detection (295 nm excitation, 390 nm emission) for human plasma. Lower concentrations of the combretastatin A4 compared to the pro-drug were used partly because of its very low aqueous solubility; in

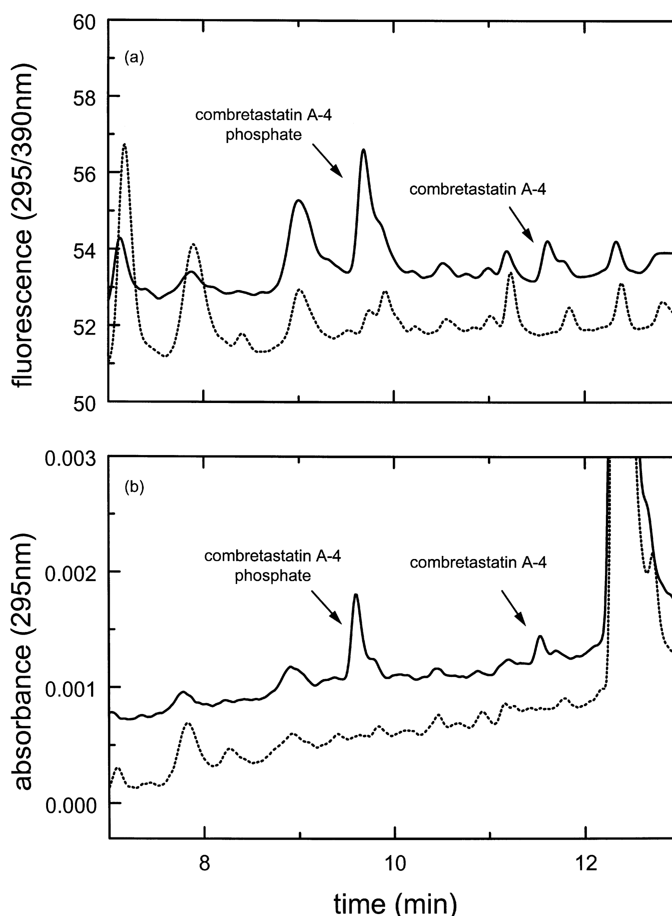


Fig. 4. Chromatograms obtained using the Hichrom RPB column of murine plasma extracts spiked with 1.4 μ*M* combretastatin A-4 phosphate and 0.35 μ*M* combretastatin A-4. Dotted line, unspiked control. (a) Fluorescence detection; (b) absorbance detection.

addition, preliminary studies had indicated that high concentrations of this drug were unlikely to be achieved in vivo. The curves were found to be linear, with correlation coefficients >0.9995 for both compounds in the two types of plasma. Extraction efficiency for both drugs using methanol as extractant was >90%. In the mouse, using absorbance detection, quantitation limits of 0.05 μ*M* were achieved, limited by interfering endogenous plasma peaks. In human plasma, using fluorescence detection, quantitation limits for combretastatin A-4 phosphate were similar to those in the mouse, and 0.0125 μ*M* for combretastatin A-4. It was important to use the highest possible grade of ion-pairing agent in the eluent in order to minimise the baseline disturbance during the gradient which otherwise affected the detection limit of the analysis.

Inter- and intra-assay precision and accuracy were determined for both murine and human plasma, using plasma spiked with known amounts of the two compounds, and the results are shown in Table 1. In order to assess the value of the method with plasma containing the drug, together with possible metabolites, blood was collected from several mice 90 min after administration of 100 mg/kg, and the plasma subjected to repeat analysis (Table 2). It is notewor-

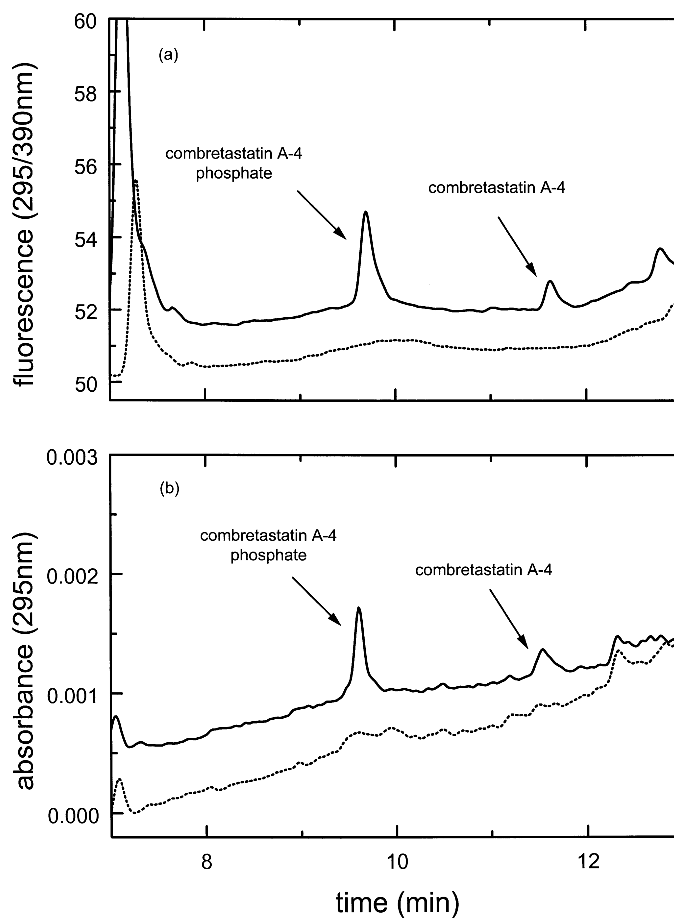


Fig. 5. Chromatograms obtained using the Hichrom RPB column of human plasma extracts spiked with 1 μ*M* combretastatin A-4 phosphate and 0.25 μ*M* combretastatin A-4. Dotted line, unspiked control. (a) Fluorescence detection; (b) absorbance detection.

Table 1
Intra- and inter-assay precision and accuracy for the determination of combretastatin A-4 phosphate and combretastatin A-4

		Concentration (μ*M*)	Intra-assay		Inter-assay	
			R.S.D. %	Accuracy	R.S.D. %	Accuracy
Mouse	Combretastatin A-4 phosphate	40	0.38 (n=4)	98.7	1.78 (n=3)	98.4
		10	3.88 (n=5)	99.2	4.43 (n=5)	102
		1	8.83 (n=5)	95.1	9.29 (n=5)	96.2
	Combretastatin A-4	10	5.86 (n=5)	100	5.25 (n=8)	101
		1	8.25 (n=5)	93.9	10.5 (n=5)	94.5
Human	Combretastatin A-4 phosphate	40	0.28 (n=4)	109	1.81 (n=4)	107
		10	3.45 (n=5)	105	1.35 (n=4)	103
		1	1.39 (n=5)	114	5.10 (n=4)	108
	Combretastatin A-4	10	2.70 (n=5)	103	2.60 (n=8)	101
		1	2.45 (n=5)	110	3.33 (n=4)	103

Table 2
Mean and standard deviation for analysis of mouse plasma for combretastatin A-4 and combretastatin A-4 phosphate 90 min after administration of 100 mg/kg of pro-drug (n=4)

	Concentration (μ*M*)	
	Intra-assay	Inter-assay
Combretastatin A-4 phosphate	2.88±0.04	2.83±0.11
Combretastatin A-4	2.81±0.02	2.76±0.11

thy that more than 1 h after administration of the drug, concentrations of the combretastatin and the pro-drug are comparable. For comparison, after administration of the phosphate ester of the anti-cancer drug etoposide in man, plasma concentrations of the pro-drug are not detectable within 10 min [8]. This suggests that combretastatin A-4 phosphate is a poor phosphatase substrate, since murine plasma phosphatase activity is normally higher than in man.

Fig. 6 shows chromatograms of a plasma extract from a mouse sacrificed 90 min after dosing with 100 mg/kg combretastatin A-4 phosphate, together with a control plasma extract, with absorbance detection at 295nm. It shows that both the parent compound and the pro-drug are readily detectable, in addition to a number of drug-related peaks. The presence of the metabolites led to the need to use the relatively high $TBAHSO_4$ concentration of 15 m*M*. Lower concentrations resulted in the co-elution of the phosphate ester and the major metabolite that elutes just before it. The ability of $TBAHSO_4$ to resolve these peaks is a result of the fact that the metabolites are of the neutral combretastatin A4 rather than of the ester. This was confirmed by incubating heparinised plasma with additional alkaline phosphatase (Fig. 7). All of the combretastatin A4 phosphate peak is cleaved and converted to combretastatin A4, showing that no other drug-related peak co-elutes with the ester, while the two main metabolites eluting before the ester are unaffected. The absorption spectra of most of the drug-related peaks are very similar, and the loss of the

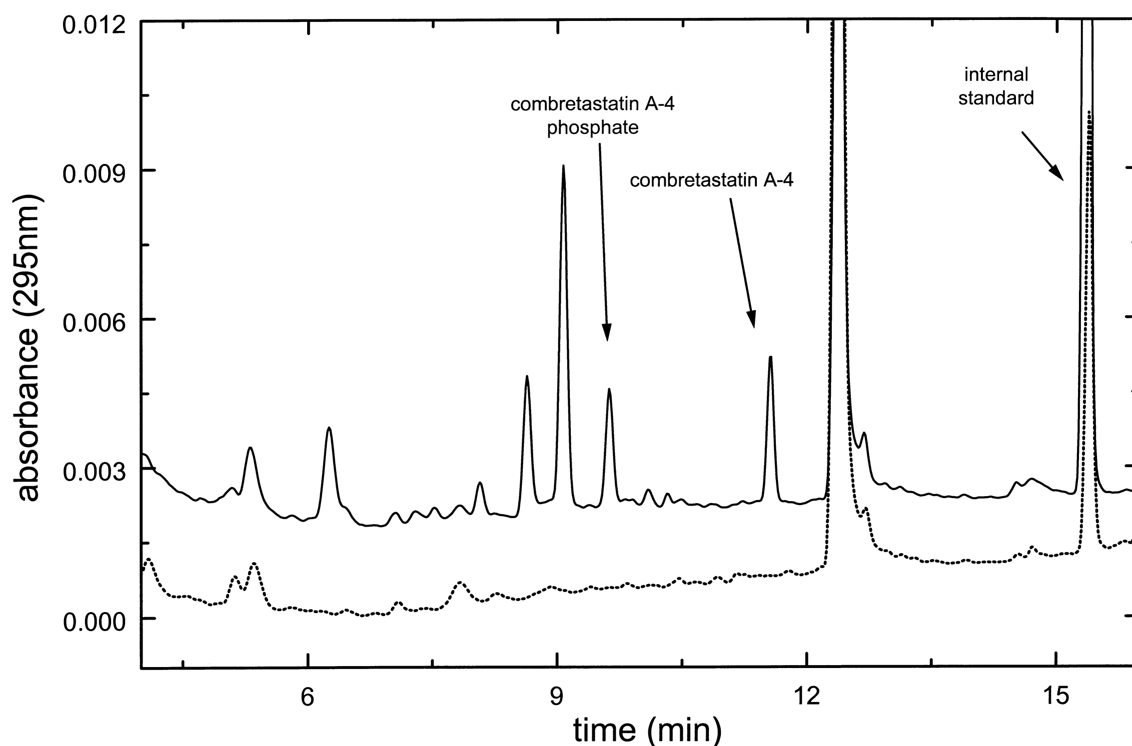


Fig. 6. Chromatogram obtained using the Hichrom RPB column of murine plasma taken 90 min after administration of 100 mg/kg combretastatin A-4 phosphate. Dotted line, control plasma.

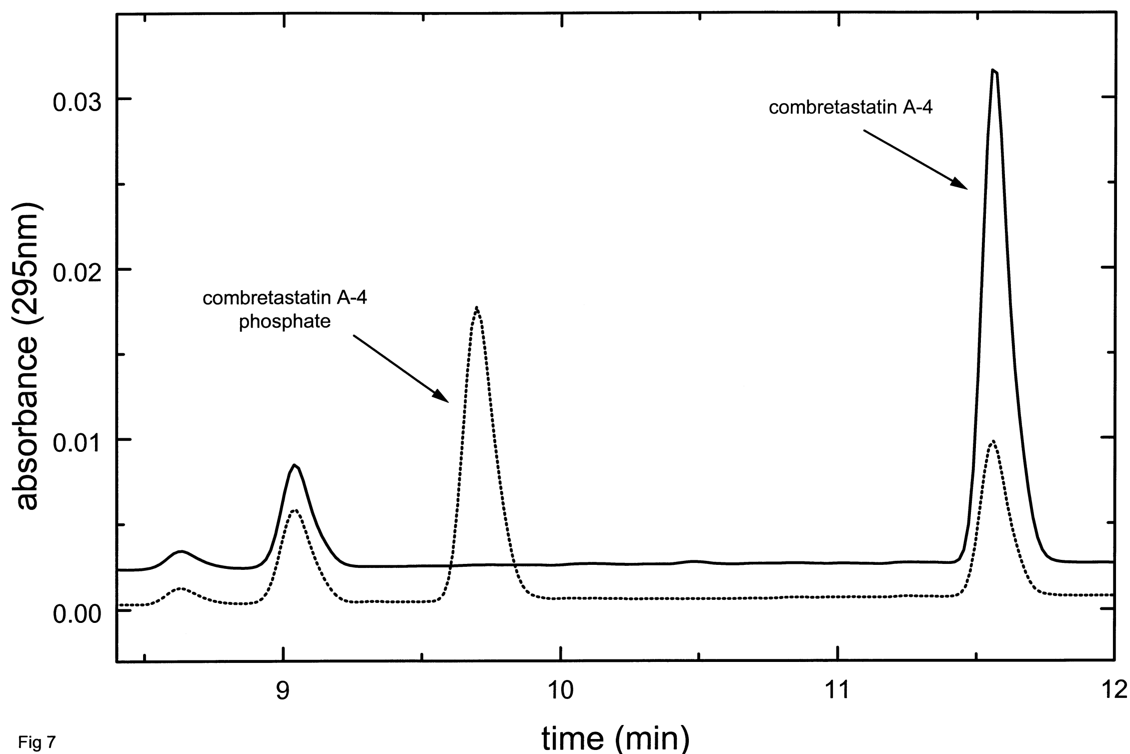


Fig. 7. Chromatogram obtained using the Hichrom RPB column of plasma from a mouse dosed with 100 mg/kg of combretastatin A-4 phosphate, incubated with alkaline phosphatase (0.1 units, 4 h, 37°C). Dotted line, plasma without enzyme.

phosphate group has little effect on the spectrum. However, any conversion to the *trans*-isomer results in a significant spectral shift, in addition to a large increase in fluorescence. These two factors should allow the ready detection of any significant amount of *trans*-products, and preliminary spectral evidence suggests that at least two *trans*-isomeric metabolites are produced. However, extreme care needs to be taken with this drug to prevent light-induced isomerisation of the *cis*-form. This was an important consideration in the development of the assay; namely that any significant conversion of the drug either before or after administration should be readily detectable.

4. Conclusions

The method described in this paper permits the determination of combretastatin A-4 and its phosphate ester in both murine and human plasma using absorbance and fluorescence detection respectively, while separating the main metabolites detected in the mouse. The ability to use the sensitivity and specificity provided by fluorescence detection should be beneficial in defining the pharmacokinetics for the forthcoming Phase I clinical trial of combretastatin A-4 phosphate.

Acknowledgements

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