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Quantitative determination of the anticancer prodrug combretastatin A1 phosphate (OXi4503, CA1P), the active CA1 and its glucuronide metabolites in human urine and of CA1 in plasma by HPLC with mass spectrometric detection

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ABSTRACT

Validated methods for the determination of CA1, the active agent derived from the prodrug CA1P, in human plasma and urine, and of CA1P and three glucuronides CA1G1, CA1G2 and CA1DG in human urine were developed using LC–MS. Plasma CA1 was extracted using solid phase extraction and validated over the range 5–1000 nM. Urine samples were analysed without extraction, and the assays validated over the range 50–2000 nM (CA1P), 25–2000 nM (CA1), 50–40,000 nM (CA1G1 and CA1G2) and 25–4000 nM (CA1DG). The mean correlation coefficient (r^2) was ≥0.997 for all assays. The intra-day and inter-day accuracy and precision were within the generally accepted criteria for bioanalytical methods (<15%). Mean recovery of CA1 from plasma was 101%, and 97% from urine. Mean urine recovery of CA1P was 98%, CA1G1 96%, CA1G2 93% and CA1DG 93%. The method was applied to plasma and urine samples from a recently completed clinical trial of the prodrug. Peak plasma concentrations of up to 470 nM CA1 were seen. The majority of drug-related material measured in urine comprised of the two monoglucuronides; CA1 and the diglucuronide were about 10-fold lower. No CA1P was detectable in urine.

1. Introduction

The combretastatins are a class of anti-cancer agents originally isolated from the African Bush Willow [1] which act by selectively depolymerising tubulin in the tumour vasculature resulting in vascular shutdown [2,3]. Combretastatin A4 phosphate (Zybrestat, fosbretabulin, CA4P) was the first of this class of agent to enter clinical trials [4,5], and an analogue, combretastatin A1 bisphosphate (OXi4503, CA1P), has recently completed a Phase I trial. Both of these agents are prodrugs which require removal of the phosphate group(s) to give the active CA4 and CA1 respectively. We have previously published a method for the simultaneous quantitation of both CA4 and CA4P in plasma by HPLC with post-column photolysis and fluorescence detection [6], and subsequently validated this for use in the clinical trial, where we also measured the glucuronide of CA4, a major metabolite found in both plasma and urine. Unlike CA4, and also CA1P, CA1 is only weakly fluorescent after photolysis [7], and although we had successfully used electrochemical detection to measure the readily oxidised catechol CA1 in mouse plasma, this was not sufficiently sensitive for the planned low starting dose in the trial, and therefore it was decided to develop a method using LC–MS detection. Initially we attempted to validate a single method which would allow us to quantify both CA1P and CA1 in plasma by LC–MS; however, the conflicting requirements of both the extraction and chromatography of CA1P and CA1 meant we were unable to measure both drugs in the same assay. In addition it was difficult to reliably measure plasma CA1P even on its own by LC–MS. We therefore validated a method for CA1P alone using post-column photolysis and fluorescence detection which had the required sensitivity, and details are published elsewhere; plasma glucuronides were also measured using a minor modification of this fluorescence assay [8,9]. There is limited non-validated pre-clinical data on the determination of CA1 and its metabolites using LC–MS in the literature [10]. The present paper describes a fully validated method for the quantitation of CA1 in human plasma and urine using LC–MS, and discusses the precautions necessary to achieve acceptable data. In contrast to plasma, we were also successful in validating separate LC–MS methods for determining CA1P in human urine, and a method for three glucuronide metabolites of CA1 (CA1G1, CA1G2 and CA1DG), and these data are also presented here.

2. Experimental

2.1. Chemicals

Structures of the compounds used are shown in Fig. 1. CA1P (OXi4503) and CA1 (OXi4500) were from Evotec (Abingdon, UK), and the glucuronides CA1G1, CA1G2 and CA1DG were from

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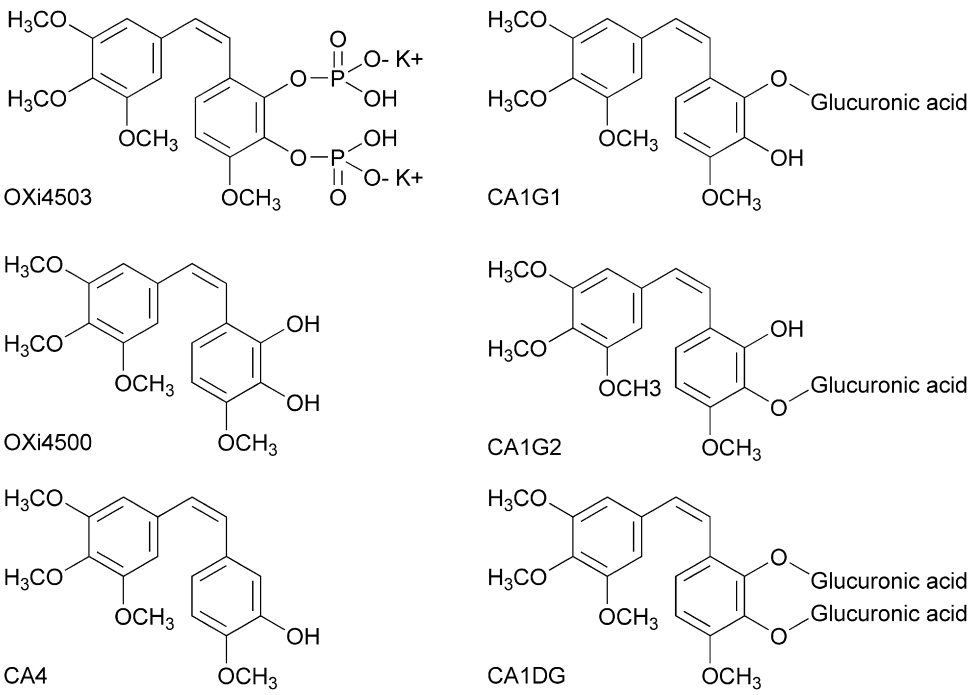

Fig. 1. Structures of the compounds studied.

Quintiles, Kansas City, MO, USA. CA4 was from Pharm-Eco Laboratories (Devens, MA, USA). Methanol and acetonitrile (LC–MS grade) and ammonium hydrogen carbonate and ammonia were from Fisher (Loughborough, UK), ammonium formate (LC–MS grade) and sodium ascorbate were from Fluka (Poole, UK) ascorbic acid was from VWR (Lutterworth, UK) and formic acid, deferoxamine (DFX) and dimethylsulphoxide (DMSO) were from Sigma (Poole, UK).

2.2. Stock solutions

All analyte standards were made initially at 1 mM. CA1 was prepared in DMSO, then diluted to 50 μM in 25% DMSO, both solutions containing 5 mM ascorbic acid. CA1G1 and CA1DG were prepared in water, CA1G2 in DMSO as it was subject to slow and variable *cis–trans* isomerisation in water. Plasma standards were prepared by diluting CA1 appropriately into human EDTA plasma containing 1 mM ascorbic acid. CA4 (internal standard, IS) was made up as a 1 mM solution in DMSO, diluted to 50 μM in 30% DMSO, then diluted to a working concentration of 2.5 μM in 30% methanol/water. Urine standards were prepared by diluting either CA1 or the glucuronides appropriately in human urine containing 1 mM ascorbic acid. DFX was prepared as a 5 mM solution in 20% methanol; 1 mL of this solution was applied to a Strata-X SPE cartridge (Phenomenex, UK) conditioned with 1 mL methanol and 1 mL 20% methanol/water. DFX was unretained, but potential interfering impurities remained on the column. The cleaned-up DFX solution was then diluted ten fold in water to give a 0.5 mM working solution.

2.3. HPLC

The HPLC system used for the studies consisted of a 2695 separations module and an EMD mass detector (ZQ2000 for CA1P) (Waters, Watford, UK). Column temperature was maintained at 35 °C, and for all but determination of CA1P in urine the flow rate was 0.45 mL/min; a flow splitter constructed of a short length of 0.007″ i.d. PEEK tubing attached to a tee fitting on the MS inlet diverted ~ half of this to waste, the remainder going to the MS detector. A divert valve (Rheodyne MX7900, Hichrom, Reading, UK) was used to divert flow away from the MS at the beginning and end of each chromatogram. For CA1P in urine the flow rate was 0.25 mL/min and no flow splitter was used.

2.3.1. CA1 (plasma)

Chromatographic separation took place on a Gemini 3 μm C18 column (150 mm × 3 mm) (Phenomenex, Macclesfield, UK). Eluent A was 20% methanol, 10 mM ammonium formate, B was 10 mM ammonium formate in methanol and C 100% acetonitrile. Initial conditions were 90% A, 10% B, with a linear gradient to 20% A, 80% B over 12.5 min, then to 100% C over 0.5 min, held for 2 min before returning to the initial conditions. The EMD was run in positive electrospray mode, with the capillary set at +2.5 kV, the desolvation gas flow 400 L/h, the cone gas flow 80 L/h, the desolvation temperature 425 °C, and the source temperature 115 °C. The dwell time was 0.4 s. CA1 was detected at *m/z* 333 (M+H) in single ion mode, with a cone voltage of 31 V at 12.2 min. The molecular weight of the IS CA4 is 316, but it was detected at *m/z* 286 (corresponding to loss of a methoxy group), using a relatively high cone voltage (55 V), at 13.7 min.

2.3.2. CA1 (urine)

Chromatographic separation took place isocratically on a Sunfire 3.5 μm C18 column (150 mm × 3 mm) (Waters, Watford, UK), eluent 50% 8 mM ammonium formate, 2 mM formic acid, 10% methanol and 40% acetonitrile. The EMD conditions were as for plasma, but with no internal standard. CA1 eluted at 5.3 min.

2.3.3. CA1P (urine)

Chromatographic separation took place on a Gemini NX 3 μm C18 column (150 mm × 2 mm) (Waters, Watford, UK). Eluent A was 5 mM ammonium hydrogen carbonate adjusted to pH 9.0 with NH_3, and B was 100% methanol. Initial conditions were 98% A, 2% B with a linear gradient to 40% A, 60% B in 8 min. The ZQ2000 was run in negative electrospray mode, with the capillary set at −1.0 kV, cone voltage 50 V, the desolvation gas flow 450 L/h, the cone gas flow 90 L/h, the desolvation temperature 450 °C, and the source

temperature 140 °C. The dwell time was 0.4 s. CA1P (8 min) was detected in single ion mode at *m*/*z* 393 (M−H).

2.3.4. CA1G1, CA1G2, CA1DG (urine)

Chromatographic separation took place on a Sunfire 3.5 μm C18 column (150 mm × 3 mm). Eluent A was 9 mM ammonium formate, 1 mM formic acid, B was 100% methanol and C 100% acetonitrile. Initial conditions were 85% A, 5% B, 10% C with a linear gradient to 10% A, 80% B, 10% C over 12 min. The EMD was run in negative electrospray mode, with the capillary set at −2.1 kV, cone voltage 33 V, the desolvation gas flow 400 L/h, the cone gas flow 80 L/h, the desolvation temperature 425 °C, and the source temperature 115 °C. The dwell time was 0.4 s. CA1DG (7.8 min) was detected in single ion mode at *m*/*z* 683 (M−H), and CA1G1 (10.5 min) and CA1G2 (11.2 min) were detected at *m*/*z* 507 (M−H).

2.4. Plasma samples

Blood from healthy volunteers or patients entered into the OXi4503 trial was collected into EDTA which inhibits plasma phosphatase activity [11]. It was spun at 1500 g for 10 min at 4 °C and 1 mL plasma transferred to a polypropylene tube containing lyophilised ascorbic acid sufficient to give a final ascorbic acid concentration of 1 mM. The latter inhibits oxidation of CA1 and the *cis–trans* isomerisation of CA1G2 [9].

2.5. Urine samples

Patient urine was collected over 0–8 and 8–24 h with sufficient added ascorbic acid to give a final concentration of >0.5 mM. For healthy volunteers, ascorbic acid was added to give a final concentration of 1 mM.

2.6. Sample preparation

As the combretastatins are somewhat light-sensitive, all procedures were carried out under reduced light.

2.6.1. Plasma CA1

To thawed standards or patient plasma samples (50 μL) was added 20 μL IS (CA4, 2.5 μM) and the sample mixed. 1 mL 10 mM HCl was added and the mixed sample was immediately applied at ∼2 mL/min to a solid phase extraction (SPE) cartridge (Strata-X, 30 mg/1 mL, Phenomenex) pre-conditioned with 1 mL methanol and 1 mL 10 mM HCl. The cartridge was washed with 1 mL 5% methanol/water, and CA1 was then eluted with 1 mL methanol (containing 100 μM sodium ascorbate) into a 4 mL amber shell vial (Alltech, Carnforth, UK) containing 10 μL of 0.5 mM DFX, and dried in a centrifugal evaporator. The sample was reconstituted in 100 μL 40% methanol, 10 mM ammonium formate, transferred to an HPLC vial (300 μL glass microinsert in a 2 mL amber snapcap vial (Jaytee Bioscience, Whitstable, UK)) and 25 μL was injected onto the HPLC.

2.6.2. Urine

Samples were analysed unextracted, and without addition of internal standard. After thawing, samples were centrifuged (2000 × *g*, 10 min), and 100 μL placed in an HPLC vial as above for analysis. For CA1P, samples were diluted 1:1 with eluent A.

2.7. Validation methods

2.7.1. Linearity

Calibration curves constructed from control human plasma or urine spiked with known amounts of the analytes were run and analysed using the HPLC software (Empower, Waters), weighted by 1/(concentration)^2. For plasma CA1, the ratio of the CA1 peak area to the IS area was plotted; for the remainder, the response at the individual mass was used.

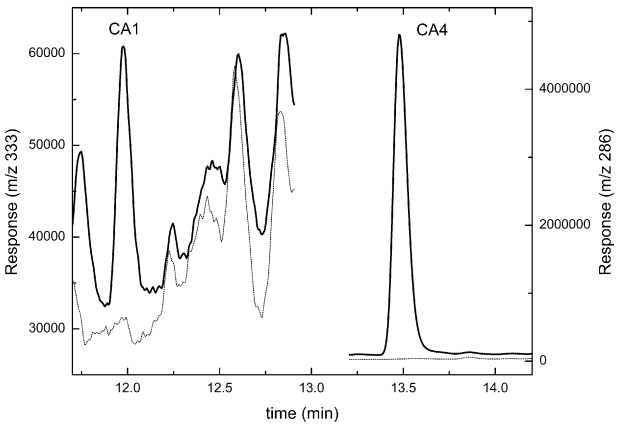

Fig. 2. Positive electrospray chromatograms of control plasma (lower line) and plasma spiked with 5 nM CA1 and IS (upper line). CA1, response at *m*/*z* 333; CA4, response at *m*/*z* 286.

2.7.2. Accuracy and precision

The intra-day precision and accuracy were determined by calculating the relative standard deviation (RSD %) and relative error (RE %) of the measurement of four replicates of each of the four validation standard concentrations analysed on the same day. Using separate samples, this was repeated on three (glucuronides) or four (CA1, CA1P) separate occasions to give the inter-day values.

2.7.3. Recovery

Recovery was determined at three concentrations (two for CA1 and CA1P in urine) by comparing the peak areas of each analyte in human urine (CA1 also in plasma) with those of equivalent aqueous solutions.

2.7.4. Stability

Stability of the analytes in plasma and urine was studied at a number of temperatures and after repeated freeze/thaw cycles. Stability of plasma CA1 was determined at 3 concentrations, and for all the analytes in urine at two concentrations.

3. Results and discussion

3.1. Selectivity

Twelve control plasma samples from different sources were extracted and none showed any significant interference where either CA1 or the IS eluted. Fig. 2 shows a typical example of a control chromatogram, along with a plasma extract spiked with 5 nM CA1 and containing the IS. Samples of human urine from ten different sources were similarly checked for interferences with CA1P, CA1, CA1G1, CA1G2 or CA1DG analysed under appropriate conditions for each analyte. Examples of control chromatograms, along with one at the LOQ for each analyte, are shown in Fig. 3A–C.

3.2. Linearity

All except that for CA1 in urine were found to be linear over the validated ranges. CA1 in urine gave acceptable QC data up to 1000 nM with a linear fit, but by using a quadratic model, the curve could be extended to 2000 nM to cover all our stability QCs. The deviation from linearity was slight, with the x^2 parameter being around an order of magnitude less than the *x* component. The data

Table 1
Calibration curve data for the analytes studied.

Analyte	Slope	%RSD	r^2	Intercept	Range
CA1 (plasma)[a]	2.01	8.50	0.997	0.0024	0.005–1 μM
CA1 (urine)[a]	4.68×10^6	11.9	0.999	−7830	0.02–1 μM[d]
CA1P[c]	3.04×10^5	3.04	0.997	1456	0.05–2 μM
CA1G1[b]	6.70×10^5	4.9	0.999	1706	0.05–50 μM
CA1G2[b]	6.62×10^5	5.4	0.999	3443	0.05–50 μM
CA1DG[b]	1.65×10^5	3.0	0.998	-23	0.02–5 μM

[a] $n = 4$.
[b] $n = 3$.
[c] $n = 6$.
[d] Validation extended to 2 μM using quadratic fit.

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

Intra-assay				Inter-assay			
Nominal conc (μM)	Calc mean conc (μM)	%RSD	%RE	Nominal conc (μM)	Calc mean conc (μM)	%RSD	%RE
CA1 plasma[a]							
500	518	3.94	3.5	500	491	5.65	−1.86
125	122	2.70	−2.2	125	124	3.28	−0.80
25	24.7	3.67	−1.4	25	25.3	9.71	1.00
5	5.24	9.56	4.8	5	5.13	13.6	2.55
CA1 urine[a]							
1500	1350	2.53	−10.2	1500	1480	9.14	−1.4
500	445	1.71	−11.0	500	499	7.98	−0.31
100	88	2.72	−12.5	100	99.8	9.05	−0.26
30	27	1.83	−9.2	30	30.3	8.66	1.08
CA1P[a]							
1600	1472	0.94	−8.00	1600	1509	2.49	−5.69
800	718	0.82	−10.3	800	737	2.03	−7.88
200	171	1.58	−14.5	200	177	3.35	−11.5
60	49.1	3.45	−18.2	60	52.0	9.47	−13.3
CA1G1[b]							
40	40.9	0.771	2.25	40	41.4	1.27	3.56
8	7.94	1.00	−0.8	8	8.32	3.67	3.96
0.8	0.801	0.772	0.09	0.8	0.814	3.47	1.78
0.06	0.056	1.38	−7.08	0.06	0.062	8.64	3.61
CA1G2[b]							
40	44.1	0.58	10.3	40	41.7	4.65	4.25
8	8.57	0.74	7.2	8	8.45	1.71	5.60
0.8	0.855	0.53	6.81	0.8	0.850	2.23	6.25
0.06	0.066	4.42	10.0	0.06	0.065	4.21	8.75
CA1DG[b]							
4.0	3.80	0.66	−5.1	4.0	3.83	2.29	−4.31
1.0	0.981	3.00	−1.9	1.0	0.968	3.65	−3.17
0.3	0.281	2.33	−6.5	0.3	0.291	4.77	−2.92
0.03	0.029	3.04	−5.0	0.03	0.030	5.03	−1.67

[a] Intra-assay, $n = 4$; interassay, $n = 16$.
[b] Intra-assay, $n = 4$; interassay, $n = 12$.

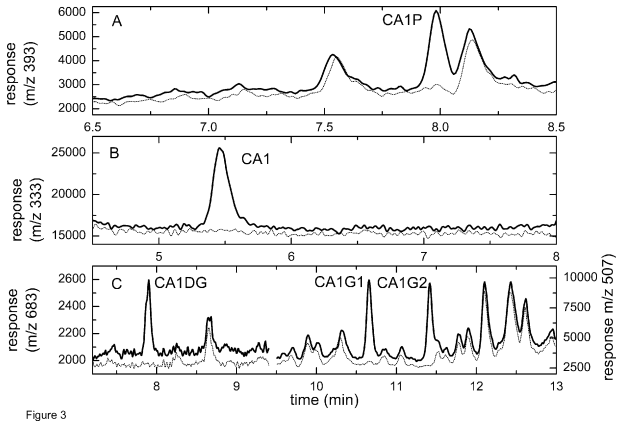

Fig. 3. Negative electrospray (CA1, positive) chromatograms of control human urine and urine spiked with the analytes. Panel A: CA1P, 50 nM, m/z 393. Panel B: CA1, 20 nM, m/z 333. Panel C: CA1DG, 20 nM, m/z 683.

is summarised in Table 1. All the correlation coefficients (r^2) were ≥0.992.

3.3. Accuracy and precision and LLOQ

For urine CA1P, the accuracy of the lowest QC (18.2%) defined this as the LLOQ; for the other analytes, precision and accuracy were within ±15%, and the LLOQ was defined as the lowest QC sample for each analyte (Table 2).

3.4. Recovery

The recovery in replicate samples was acceptable, ranging from 82 to 104% (Table 3). Although there was some evidence for a small reduction in recovery of the glucuronides at low concentrations, the QC data, which extends to substantially lower concentrations, does not indicate this has any significant impact on the accuracy of the method. For the urine samples, run without extraction, recovery samples also served to demonstrate an absence of matrix effect.

Table 3
Recovery of the analytes.

Drug	Concentration	Recovery (%)	%RSD
CA1 (plasma)[a]	500	99.2	7.2
	125	104	5.0
	25	101	5.5
CA1 (urine)[a]	1500	100	1.4
	100	93.7	1.8
CA1P (urine)[b]	1600	97.9	2.3
	200	99.5	1.7
CA1G1 (urine)[b]	40	103	1.2
	8.0	97	0.7
	0.8	87	2.0
CA1G2 (urine)[b]	40	99	0.9
	8.0	91	0.5
	0.8	88	2.8
CA1DG (urine)[b]	4.0	98.6	2.4
	1.0	99.4	0.4
	0.23	82	1.7

[a] $n = 4$.
[b] $n = 3$.

Matrix effect in plasma was assessed by infusing drug directly into the mass spectrometer while running a plasma blank [12]. Fig. 4 shows chromatograms obtained from injections of a control plasma extract either with (solid line) or without (dotted line) continuous post-column infusion of either CA1 or CA4. No evidence for significant suppression or enhancement of the mass spectrometer signal was seen for either analyte in the region in which each elutes. In addition, no significant differences were observed in QC samples from three separate sources.

3.5. Stability

Stability of the analytes in plasma and urine was studied at a number of temperatures and after repeated freeze/thaw cycles. Stability of plasma CA1 was determined at 3 concentrations, and for all the analytes in urine at two concentrations. All were shown to be stable in the autosampler for 24 h, at room temperature for 4 h, and at −80 °C for at least one year (CA1 plasma), four years (CA1 and CA1P in urine), and at least 42 weeks (CA1DG, CA1G1 and CA1G2 in urine) where deviation from the nominal concentrations was <15%. All were also stable after three freeze–thaw cycles.

3.6. Method development

3.6.1. Plasma CA1

As a catechol, CA1 is susceptible to oxidation. It was therefore critical to the success of the plasma assay of CA1 to prevent loss through oxidation to the corresponding reactive quinone, which may go on to form other adducts with nucleophiles such as thiols [13], at any stage during the analytical process. The drug proved particularly susceptible to oxidation after extraction using simple protein precipitation techniques with acid or acetonitrile which we attributed to the release of bound iron which can catalyse the oxidation process. The aim of the method was therefore to maintain reducing conditions while minimising the scope for heavy-metal catalysed degradation. This was achieved in a number of ways. The anti-oxidant ascorbic acid was included in the plasma samples and during the extraction; although in theory this could have resulted in artificially elevated levels of CA1 due to reduction of any free quinone in the samples, in practice, the concentration of this reactive species would be expected to be very low due to further reactions alluded to above. Samples were subjected to SPE immediately after addition of the acid diluent which minimised the risk of oxidation by released iron, followed by a wash step designed to remove any metals. Elution took place in the presence of sodium ascorbate, and the elution vials contained the chelator DFX to bind any remaining metals. The latter had the added benefit of significantly improving the peak shape of the CA1, perhaps due to masking of free metal sites on the column which otherwise resulted in adsorption of the drug.

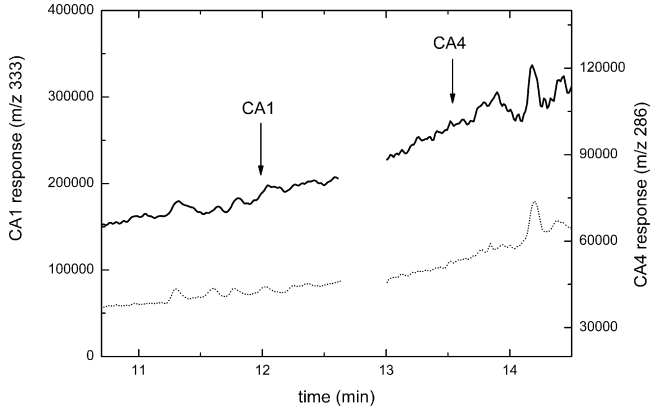

Fig. 4. Chromatograms obtained from injections of a control plasma extract either with (solid line) or without (dotted line) continuous post-column infusion of either CA1 or CA4. Retention times of the two analytes are marked.

Chromatographic conditions were chosen to ensure that known metabolites, the two possible monoglucuronides (not validated in this method), and also the monophosphate intermediates resulting from the hydrolysis of the prodrug CA1P [8], eluted before the CA1. Although no authentic standards were available for these monophosphates, and the assay has not been validated for them, we did routinely monitor for them at m/z 413 (M+H) (cone voltage 29 V) between 8 and 10.5 min. This was the main reason for the SPE-cleanup of the DFX, as otherwise contaminants in the DFX stock co-eluted with the monophosphates. Finally, the m/z 286 fragment (corresponding to loss of methoxy) was used for the IS as some control plasma was found to contain an interference at m/z 317 (M+H).

3.6.2. Urine metabolites

For plasma CA1P, we found that reliable extraction required the presence of high concentrations of the ion pairing agent tetrabutylammonium hydrogen sulphate (TBA), and this allowed us to successfully validate an HPLC fluorescence method. However, TBA suppresses the LC–MS response, and this was a major factor in our failure to develop a method for measuring plasma CA1P with this mode of detection. In contrast, because extraction of CA1P from urine was not required, we were able, by using a high pH eluent and negative mode electrospray, which gave a good, consistent chromatographic peak, to successfully validate the assay for the prodrug by LC–MS. The glucuronides were also determined in negative mode as although they ionised well in positive mode, and could also be readily fragmented to the parent CA1 by increasing the cone voltage, measuring M−H showed the least level of interference in control urine. As samples were run unextracted, the recovery samples could also be used to demonstrate that suppression was not a problem with any of the analytes. Negative electrospray, which is less sensitive to suppression effects, was used for the more hydrophilic metabolites, while more hydrophobic compounds such as CA1 are less susceptible to these effects in positive mode [14].

3.6.3. Clinical application

More than 40 patients received escalating doses of CA1P. Plasma concentrations of CA1 did not exceed 470 nM in any of the patients up to the maximum tolerated dose, and most were much lower than this, and thus well within the validated range. In urine, no CA1P was seen, and in just two patients, the urine CA1 concentration

exceeded 2 μM and therefore required dilution. Concentrations of the diglucuronide were similar to those of CA1, while the two monoglucuronides were the main metabolites observed, with levels around an order of magnitude higher [15].

4. Conclusion

These assays have been successfully applied to the analysis of over 400 plasma samples, and approximately 80 urine samples from patients in a clinical trial of CA1P. Although the urine analyses involved three separate assays, only minimal sample preparation was required, making them quick and simple to carry out.

Acknowledgement

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