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Reversed-phase high-performance liquid chromatographic determination of the new antitumor agent cyclopentenyl cytosine in biological fluids

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Abstract

Cyclopentenyl cytosine (CPE-C) is a synthetic carbocyclic nucleoside that possesses diverse antitumor and antiviral activity. CPE-C has been studied extensively at the preclinical level and has been evaluated in a Phase I clinical trial involving patients with solid tumors. A narrow-bore, reversed-phase HPLC method that has been developed for the sensitive measurement of CPE-C in plasma and urine in order to carry out these studies is described. Covalent solid-phase extraction based on an immobilized phenylboronic acid ligand is employed to isolate both CPE-C and endogenous ribonucleosides from the biological matrix selectively and efficiently. This is followed by isocratic elution of the extract with pH 5.0, 0.1 *M* ammonium formate buffer at 0.150 ml/min on a tandem, switchable, C_{18} narrow-bore (2.1 mm I.D.) column system in which the precolumn is automatically backflushed to eliminate late-eluting components. UV detection at 278 nm provides a limit of quantitation of 0.1 μ*M* for CPE-C in rat and human plasma with a precision better than 4% for the range 1–20 μ*M* in rat plasma. Application of this assay to the determination of the bolus dose plasma kinetics and disposition of 2 mg/kg CPE-C in rats is illustrated. This method is amenable to partial automation and is well-suited for the analysis of clinical samples.

Keywords: Cyclopentenyl cytosine

1. Introduction

Cyclopentenyl cytosine [1-(3-hydroxymethyl-4,5-dihydroxy-2-cyclopenten-1-yl)cytosine, CPE-C, NSC 375575, Fig. 1] is a synthetic pyrimidine analogue of the potent antiviral and antineoplastic fermentation product neplanocin A [1–4]. CPE-C possesses a broad range of in vitro antitumor and antiviral activity [1,3,5] including the ability to induce differentiation in human promyleocytic leukemia cells at concentrations as low as 0.1 μ*M* [6]. This carbocyclic nucleoside is especially potent in mice against lines of L1210 leukemia both sensitive and resistant to arabinosyl cytosine, and produces multiple long-term survivors when administered on a

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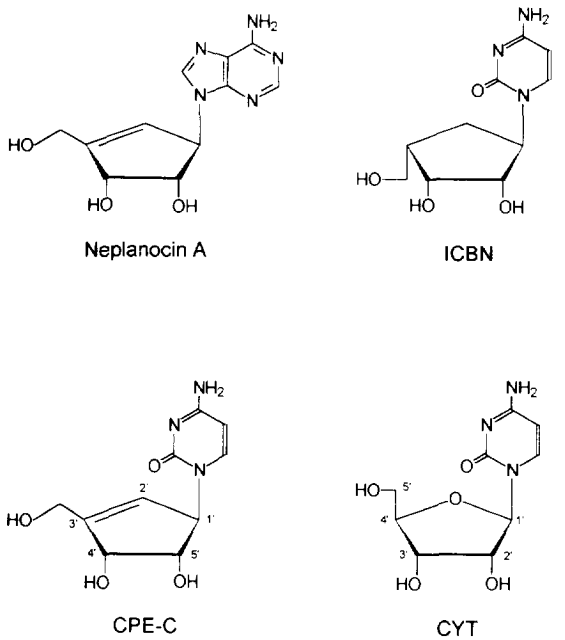

Fig. 1. Chemical structures of neplanocin A, cyclopentenyl cytosine (CPE-C), isocarbodine (ICBN) and cytidine (CYT). The positional numbering of the cyclopentenyl and ribofuranosyl rings is indicated for comparison.

chronic treatment schedule at a dose of 1 mg/kg [7]. CPE-C also shows good in vivo activity against all three of the human tumor xenograft models used in the NCI tumor panel [8].

The activity of CPE-C appears to result from its phosphorylation to the corresponding triphosphate with the subsequent inhibition of CTP synthetase, a rate-limiting enzyme in de novo pyrimidine biosynthesis [6,7,9,10]. When compared to 3-deazauridine, a well-characterized inhibitor of CTP synthetase, CPE-C possesses a wider spectrum of antitumor activity and is 100 times more potent [8]. CPE-C also functions as a competitive inhibitor of uridine-cytidine kinase to reduce UMP and CMP levels and thus intensify the depletion of CTP [1]. CPE-C is also a poor substrate for both cytidine deaminase and pyrimidine nucleoside phosphorylase [1], so this compound should possess a favorable duration of action since it will not be metabolically inactivated rapidly.

Because of its activity and favorable chemical and enzymatic properties, the NCI has undertaken the clinical development of CPE-C. Accordingly, selective and sensitive methods have been required to measure this drug in biological samples in order to carry out necessary preclinical and clinical pharmacology studies. This report describes our development of suitable HPLC methodology to measure CPE-C in plasma and urine at concentrations which correspond to those of in vitro activity (0.1–10 μM).

2. Experimental

2.1. Materials

Cytidine, uridine, pseudouridine and adenosine were purchased from Aldrich (Milwaukee, WI, USA), while pH 7.4 phosphate-buffered 0.9% sodium chloride solution was obtained from Advanced Biologies (Silver Spring, MD, USA). CPE-C was of pharmaceutical purity and was acquired from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI. Isocarbodine (Fig. 1), which was used as an internal standard, was synthesized by catalytic hydrogenation of CPE-C [11]. All other chemicals and solvents were reagent grade and were used without further purification.

2.2. Sample preparation

Buffer, plasma and urine samples were generated as described in subsequent sections. Isocarbodine was employed as an internal standard; and 10 μl of a 100 μM solution (1 nanomole) was added to 0.25 ml buffer sample, 0.50 ml plasma sample or 0.50 ml diluted (20×) urine sample with a microliter syringe and briefly vortexed to thoroughly mix. Urine samples were analyzed directly by HPLC without further treatment. One milliliter Bond Elut phenylboronic acid (PBA) covalent solid-phase extraction cartridges containing 100 mg sorbent (Analytichem International, Harbor City, CA, USA) were washed twice with 1.0 ml methanol and then activated by addition of 2×1.0 ml 0.1 M, pH 10 phosphate buffer. The entire buffer sample was loaded directly on the activated PBA cartridge. Plasma samples were ultrafiltered in a Centrifree micropartition system (Amicon, Danvers, MA, USA) by centrifugation at 1500 g for 25 min. A 0.25-ml aliquot of the resultant ultrafiltrate

was added to the activated PBA cartridge. Samples were slowly pushed through the PBA cartridge with a positive N_2 pressure, and the cartridge washed with an additional 2×1.0 ml 0.1 *M*, pH 10 phosphate buffer. Nitrogen gas was then used to remove as much of the buffer wash as possible from the cartridge. The compounds of interest were eluted with 0.25 ml 2 *M* formic acid at a flow-rate of approximately 0.10 ml/min using a positive N_2 pressure if required. The formic acid eluant was neutralized with approximately 50 mg of solid NH_4HCO_3, and the carbon dioxide that was formed was removed by a combination of vortexing and sonicating for 10 min at room temperature. A 10-μl aliquot of the neutralized sample was subjected to HPLC analysis as described in Section 2.3.

2.3. Chromatographic system

Chromatographic analyses were carried out on an HPLC system consisting of a Gilson 401/231 autosampler, an LKB 2150 pump for primary solvent delivery and a Waters 6000A pump for precolumn backflushing, a Valco EC6U electronic switching valve and a Gilson 116 variable-wavelength UV detector. Peak areas were integrated using a Spectra-Physics Chromstation-AT chromatography data system (Spectra-Physics, San Jose, CA, USA) consisting of a SP4200 computing integrator connected to a Compaq 386, Model 70 microcomputer (Compaq, Houston, TX, USA). Automatic operation was effected through an LKB 2152 HPLC controller, which controlled the primary pump and provided contact closures to initiate sample injection, integration and valve switching to backflush the precolumn (Fig. 2).

Components of interest were separated on narrow-bore C_{18} reversed-phase columns connected in tandem and eluted with pH 5.0, 0.1 *M* NH_4HCO_2 buffer at a flow-rate of 0.150 ml/min. Two Brownlee (Applied Biosystems, Santa Clara, CA, USA) 2.1× 30 mm ODS cartridges in a 60 mm column holder served as a precolumn, while a 2.1×250 mm Supelco (Bellefonte, PA, USA) LC-18S column was used for the analytical separation (Fig. 2A). This column system required an initial conditioning with 20–30 column volumes of methanol followed by a slow, 1-h linear gradient to 0.1 *M*, pH 5.0 ammonium formate buffer and subsequent equilibration

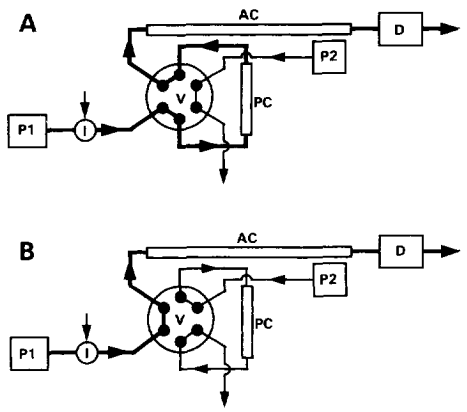

Fig. 2. Column and switching valve configuration for (A) dual column separation and (B) precolumn backflushing. P1 = High-pressure pump and system controller, I = injection valve and sample loop, V = 6-position automated switching valve, PC = cartridge precolumn, P2 = high pressure pump, AC = analytical column and D = variable-wavelength UV detector.

with this aqueous mobile phase for an additional 3 h. Adequate retention and resolution of the components of interest were preserved as long as column flow was maintained. When the nucleosides of interest had eluted from the precolumn, the switching valve was automatically activated so that the precolumn was backflushed with mobile phase at a flow-rate of 0.15 ml/min (Waters 6000A pump) while the analytical column elution remained constant at 0.150 ml/min (Fig. 2B). When the final component had eluted from the analytical column, the valve was switched again so that precolumn and analytical column were in series and the system could equilibrate for 5 min before the next injection.

2.4. Hydrolytic stability of CPE-C and isocarbodine

The stability of CPE-C was evaluated in both buffer and plasma at 0.5, 4.0 and 20 μ*M* concentrations. Appropriate concentrations of CPE-C were incubated at 37°C in 10.0 ml each of pH 1, 0.1 *M* KCl buffer; pH 6.0, 0.1 *M* phosphate buffer; pH 7.4 phosphate buffered 0.9% NaCl solution; and pH 10.0, 0.1 *M* phosphate buffer. Samples (0.25 ml) were taken from each incubated buffer solution for analysis at 0, 0.5, 1, 2, 4, 6 and 24 h, with the pH 1.0 and pH 10.0 samples being neutralized before work-

up and analysis. Blood was obtained from untreated male Sprague–Dawley rats, untreated male beagle dogs and normal human volunteers and was collected in rubber-stoppered 10-ml glass tubes containing 143 units of USP heparin (Vacutainer, Becton Dickinson and Co., Rutherford, NJ, USA). The heparinized blood was immediately centrifuged at 1100 *g* for 3–5 min to obtain plasma, which was either used immediately or frozen and stored at −20°C. Appropriate concentrations of CPE-C were incubated at 37°C with shaking in 5.0-ml aliquots of each type of plasma. One-half milliliter samples were removed from each incubation mixture on the same schedule as indicated for the buffer stability studies and worked up and analyzed according to the standard procedure described in Section 2.2 Section 2.3.

2.5. Plasma protein binding

The plasma protein binding of 0.5, 4.0 and 20 μ*M* CPE-C was evaluated in rat, dog and human plasma by measuring CPE-C in plasma both before and after ultrafiltration. Two sets of CPE-C spiked plasma samples, consisting of 4 replicates each, were prepared for each concentration and plasma type. After a 30-min equilibration period, one set of plasma samples was analyzed for CPE-C in the standard manner. The other set of plasma samples was first ultrafiltered, and a 0.50-ml aliquot of ultrafiltrate was taken for analysis of CPE-C. Plasma protein binding was defined as the percent difference in CPE-C plasma (C_p) and ultrafiltrate (C_u) concentration according to the following formula:

$$\%\text{Bound} = 100(1 - [C_u/C_p])$$

The plasma protein binding of the isocarbodine internal standard was determined in the same way except that absolute peak areas in ultrafiltrate and plasma were compared for the one concentration of 2 μ*M*.

2.6. Recovery, precision and accuracy

Appropriate volumes of pH 7.4 phosphate buffered 0.9% NaCl solution, control rat plasma, control dog plasma, normal human plasma and diluted (20×) control rat urine were spiked with CPE-C in the range 0–20 μ*M*. Each sample of a specific concentration was processed and analyzed in duplicate. A standard curve for each sample matrix was then constructed by generating the best straight line fit for the peak area ratio of CPE-C to isocarbodine internal standard versus CPE-C concentration using a calculator (TI-66, Texas Instruments, Dallas, TX, USA) or personal computer (GraphPAD, ISI Software, Philadelphia, PA, USA) linear least-squares program. Recovery of CPE-C and isocarbodine from individual sample matrices was evaluated by direct comparison of integrated peak areas with those from the corresponding untreated buffer standards. Assay precision was determined by quadruplicate analysis of spiked biological samples at selected concentrations.

2.7. CPE-C bolus dose pharmacokinetics in the rat

The in vivo plasma pharmacokinetics of CPE-C was determined after administering an i.v. bolus injection of 2 mg/kg CPE-C to mature male and female Sprague–Dawley rats weighing 150–300 g. This dose was equivalent to an active, yet nontoxic, dose for tumor-implanted mice [4,8]. Drug was administered at a concentration of 10 mg/ml in 0.9% NaCl solution via the tail vein. One milliliter blood samples were collected in heparinized tubes before CPE-C treatment and at the times indicated in Fig. 4 by puncture of the retroorbital plexus. Rats were transiently anesthetized with ether to the extent that the righting reflex was inhibited, for drug administration, blood sampling and sacrifice. Animals were awake and allowed free access to food and water at all other times. Plasma was separated from red blood cells by centrifugation at 1500 *g* for 10 min, and samples were treated and analyzed as described in Section 2.2 Section 2.3. A standard curve, covering the range 0.1–10 μ*M*, was constructed by spiking 0.5-ml aliquots of pooled pretreatment plasma with the appropriate amounts of CPE-C. These spiked plasma standards were analyzed for CPE-C concurrently with the regular samples.

Experimental plasma drug concentration (C_p) versus time data points were fit to a biexponential function representing a two-compartment open model ($C_p = Ae^{-\alpha t} + Be^{-\beta t} + C$) with MLAB, an on-line computer modeling program utilizing an interac-

tive, non-linear least squares program [12]. Based on observed assay characteristics, each data point was weighted by $1/(C_p)^2$. Pharmacokinetic parameters were calculated using standard equations for the two-compartment open model [13]. The area under the C_p versus time curve (AUC) was also calculated using the trapezoid rule [14] with an extrapolation to infinity from the last data point using the terminal (γ) rate constant.

2.8. Urinary excretion of CPE-C

Three mature male Sprague–Dawley rats weighing 370–400 g were administered 2 mg/kg CPE-C in the same manner as described in Section 2.7. Prior to drug administration the animals were kept in individual metabolic cages for 24 h for the collection of pretreatment urine. Following treatment with CPE-C, the animals were returned to the metabolic cages and allowed free access to food and water. Urine was collected for the period 0–8, 8–24 and 24–48 h, and the metabolic cage was washed with 3–5 ml distilled water following each collection. Urine and cage washing for the same individual sample were combined and the total volume measured. Each sample was further diluted 20× with distilled water before analysis as described above. Approximately 1 ml blood was also obtained from each lightly anesthetized animal at 8 and 24 h by retroorbital puncture and at 48 h by exsanguination. These samples were analyzed for CPE-C to determine whether plasma levels of the parent drug persisted for an extended period.

3. Results

3.1. Chromatography

Baseline separation of CPE-C, the isocarbodine internal standard and endogenous ribonucleosides (adenosine, cytidine, pseudouridine and uridine) was possible using isocratic conditions and a C_{18} reversed-phase column with a high carbon loading (Fig. 3A, Table 1). With this column, use of a mobile phase of 0.1 *M*, pH 5.0 ammonium formate buffer with no organic modifier after appropriate column conditioning was found to give the best retention and separation for the pyrimidine nucleosides of interest. The long retention of adenosine and other uncharacterized endogenous plasma components under these chromatographic conditions, however, required use of a dual component column system. In this system the first column or precolumn provided only separation of the early eluting pyrimidine nucleosides from the long-retained components, which were backflushed to waste before they could enter the analytical column. This operation was accomplished automatically by an electronically actuated six-port, two-position valve which was configured as indicated in Fig. 2. The valve switching time was determined from the difference in retention time for uridine, the latest eluting component of interest, when analyzed with the precolumn and analytical column in series (Fig. 2A) and with the analytical column alone (Fig. 2B). Narrow-bore (2.1 mm I.D.) columns were employed in this system in order to allow maximum sensitivity for small sample volumes (5–10 μl). For this diameter column, a mobile phase flow-rate of 0.150 ml/min gave acceptable resolution with minimum analysis time (total cycle time = 35 min). The dead volume in injector, column, valve and detector connections was minimized by using the shortest possible lengths of 0.010 inch (0.025 cm) I.D. stainless steel tubing.

3.2. Recovery, precision and accuracy

Isolation of the CPE-C, isocarbodine and other nucleosides of interest from plasma was accomplished by a two-step process involving ultrafiltration to remove plasma proteins and then covalent solid-phase extraction of the ultrafiltrate on a commercially available phenylboronic acid minicolumn to isolate *cis*-1,2-diols. A pH of 10 was found to be the best compromise for efficient formation of the covalent cyclic boronate complex and minimum sample decomposition. A relatively high concentration of 2 *M* formic acid was used to decompose the covalent boronate complex and elute the nucleosides of interest in a minimum volume. Recovery of CPE-C and the isocarbodine internal standard with this two-step isolation process was determined for buffer, and for plasma and urine from several species. Duplicate samples for each concentration level over the range 0.1–20 μ*M* in each matrix were

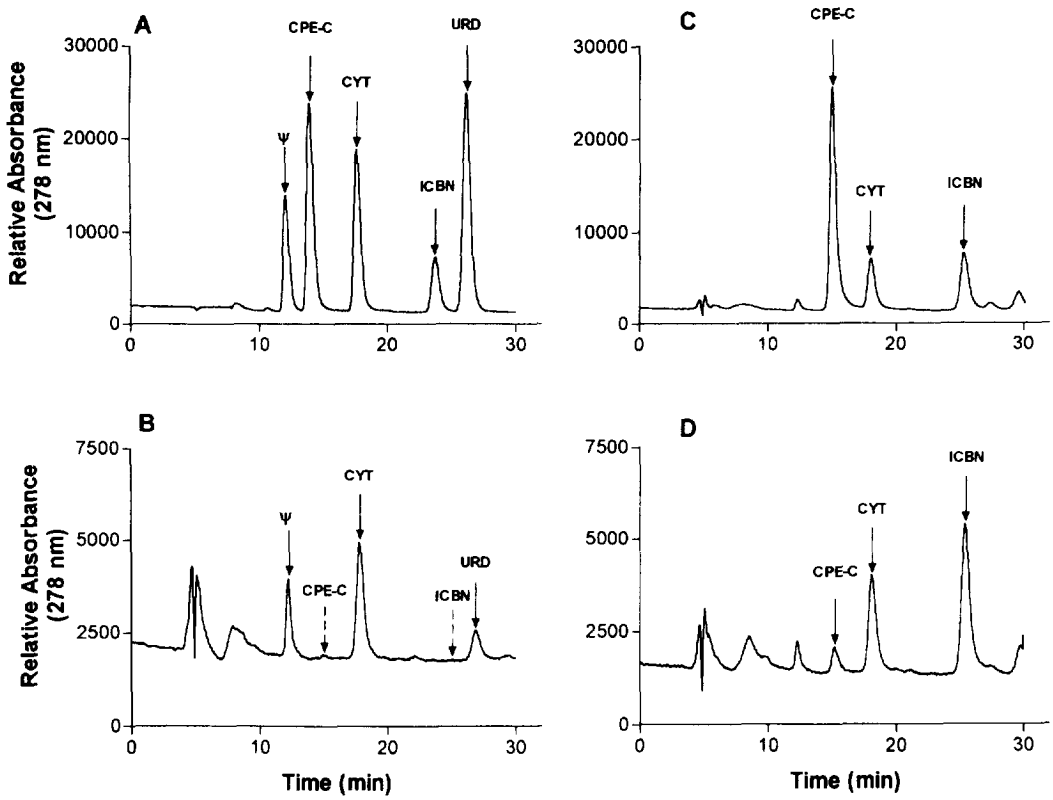

Fig. 3. Narrow-bore chromatograms of (A) standard mixture of pseudouridine (Ψ), cyclopentenyl cytosine (CPE-C), cytidine (CYT), isocarbodine (ICBN) internal standard and uridine (URD); (B) pretreatment rat plasma with dashed arrows indicating expected retention time of CPE-C and ICBN; (C) rat plasma obtained 40 min after administration of 20 mg/kg CPE-C as a bolus i.v. dose and (D) rat plasma obtained 8 h after administration of the same dose. The measured concentrations of CPE-C at 40 min and 8 h were 7.14 μg/ml (29.9 μ*M*) and 0.29 μg/ml (1.2 μ*M*), respectively. The concentration of ICBN internal standard was 10 μ*M* in chromatograms C and D.

processed and analyzed and then compared directly to the appropriate standards. Average recoveries ranged from approximately 75–85% as indicated in Table 2. Individual recoveries exhibited a slight dependence on CPE-C concentration, with plasma samples spiked at levels lower than 0.5 μ*M* typically having a recovery at the low end of the S.D. range. This same data was also used to evaluate the characteristics of the linear calibration curves that could be constructed for CPE-C in each matrix (Table 3). With the isolation procedure and chromatography system described above, the limit of quantitation (LOQ) for CPE-C ($S/N>5$ [17]) was 0.1 μ*M* (24 ng/ml) in all the matrices evaluated except for dog plasma. For this latter matrix, low level interferences, as evidenced by the positive standard curve *y*-intercept, made the LOQ equal to 0.2 μ*M*. The limit of detection ($S/N>3$) for CPE-C corresponded to one-half the LOQ in all cases.

3.3. Hydrolytic stability of CPE-C and isocarbodine

CPE-C was hydrolytically stable in all buffers except for pH 10, 0.1 *M* phosphate buffer where slow degradation was observed. No decomposition was seen at any CPE-C concentration at pH 1, pH 6 or pH 7.4 during incubation for 24 h at 37°C. At pH 10 and 37°C, 9% of the initial CPE-C concentration was lost after 1 h and 23% by 24 h. CPE-C was also

Table 1
Chromatographic and spectroscopic properties of selected nucleosides

Compound	Capacity factor (k')	λ_{max} (nm)	ϵ (mol^{-1})
Pseudouridine (Ψ)	1.40	260[a]	7900[b]
CPE-C	1.72	278[b]	8550
Cytidine	2.50	271[c]	9120
Cyclopentenyl uracil (CPE-U)	2.68	266[d]	11 750
Isocarbodine	3.60	275[e]	8670
Uridine	4.17	265[a]	7585
Inosine	6.66	249[f]	12 300
Adenosine	15.58	260[g]	14 800

[a] Ref. [15], p. 82.
[b] Ref. [8].
[c] Ref. [15], p. 81.
[d] Ref. [1].
[e] Ref. [11].
[f] Ref. [15], p. 119.
[g] Ref. [15], p. 118.
[h] Ref. [16].

Table 2
Assay characteristics for various matrices

Parameter	Compound (μM)	Measured value
Rat plasma		
Recovery[a]	CPE-C (0.1–10 μM)	75±13% (n=15)
	ICBN (2.3 μM)	78±7% (n=15)
Protein binding[b]	CPE-C (0.1–10 μM)	0–17%
	ICBN (1.7 μM)	0%
LOQ[c]	CPE-C	0.1 μM
Dog plasma		
Recovery	CPE-C (0.2–20 μM)	84±4% (n=16)
	ICBN (2.0 μM)	85±5% (n=16)
Protein binding	CPE-C (0.5–20 μM)	0–16%
	ICBN (4.0 μM)	0–11%
LOQ	CPE-C	0.2 μM
Human plasma		
Recovery	CPE-C (0.1–10 μM)	74±10% (n=13)
	ICBN (1.9 μM)	83±9% (n=13)
Protein binding	CPE-C (0.5–50 μM)	0%[d]
LOQ	CPE-C	0.1 μM

[a] Mean±S.D. for number of replicates indicated in parentheses.
[b] Range.
[c] Concentration of lowest spiked standard or concentration at which $S/N>5$.
[d] Calculated binding was less than zero, indicating concentration of sample due to removal of plasma proteins.

hydrolytically and enzymatically stable for 24 h at 37°C in rat, dog and human plasma. A less extensive evaluation of the hydrolytic stability of isocarbodine internal standard was also carried out with similar results.

3.4. Plasma protein binding

CPE-C showed no significant plasma protein binding at the three concentrations in the range 0.5–20 μM studied (Table 2). This very limited protein binding exhibited no significant measurable concentration dependence. The isocarbodine internal standard exhibited no measurable plasma binding when evaluated at the 2.3 μM concentration usually used for plasma sample analysis.

3.5. CPE-C bolus dose pharmacokinetics

CPE-C could be measured in rat plasma for 24 h following a single 2 mg/kg i.v. bolus dose. Plasma concentrations rapidly decreased from 10.4 μM at 5 min to approximately 2.1 μM at 1 h (Fig. 4). After this time CPE-C C_p decreased much more slowly, being still measurable at the 0.1 μM level at 24 h (data not shown). Pharmacokinetic analysis of the initial (0–8 h) C_p versus time data provided a good fit to a biexponential function representing a two-compartment kinetic model. This model was used to derive the α- and β-phase half-lives of 12.7 min and 1.52 h, respectively, as well as to calculate an area-under-the-curve value of 529 μM min and a total body clearance of 15.8 ml/min/kg.

3.6. Urinary excretion of CPE-C

The CPE-C concentration of the dosing solution was measured and used to calculate the percentage of the actual administered i.v. bolus dose excreted in the urine. For 2 mg/kg drug, the majority of the dose (46±8%) was excreted unchanged in the urine within the first 8 h. At 24 h the cumulative excretion of unchanged CPE-C increased to 59±10% and at 48 h was 64±9%. Measurable amounts of cyclopentenyl uracil were not observed in any collected rat urine fraction.

Table 3
Assay characteristics for rat plasma and urine
Standard curves for CPE-C

Parameter	Plasma[a]	Urine[b]
Slope	0.4234	0.9785
S.E.[c] slope	0.0199	0.00536
Intercept	−0.0520	−0.0048
S.E. intercept	0.0907	0.0231
Range (μM)[d]	0.1–10	0.1–10
Number of standards	7	7
Correlation coefficient (r^2)	0.9946	0.9999

Accuracy and precision for the measurement of CPE-C in plasma

Concentration (μM)	Measured concentration (μM)	Relative error (%)	Precision (R.S.D., %)	Number of replicates
Within-day				
1.00	0.94	6.0	3.7	5
5.00	4.81	3.9	2.4	5
20.0	19.26	3.7	2.5	5
Between-day				
2.00	2.03	1.5	0.9	4

[a] Internal standard concentration was 2.3 μM. Individual points are the mean from the measurement of at least two replicate samples.
[b] Internal standard concentration was 1.0 μM. Individual points are the average of two replicate analyses.
[c] Standard error.
[d] Concentration range over which samples were spiked.

4. Discussion

The main goal of this work was to develop an analytical method that would allow reliable measurement of CPE-C in biological fluids in the 0.1–10 μM concentration range, where in vitro antitumor activity has been observed [1,4–8]. Since the analytical method would initially be applied to preclinical pharmacology studies, there was also a need to make it amenable to the smallest sample volume possible to allow pharmacokinetics to be determined in individual small animals such as rats. Also desirable, but of secondary importance, was the ability to measure any CPE-U produced by deamination and the endogenous concentrations of cytidine and uridine, which are both biomodulators of CPE-C activity [10,18]. This required that the efficiency and selectivity of the procedures for isolating the analytes from the biological matrix be carefully considered and that the chromatographic system be optimized for maximum sensitivity. For a sample matrix such as plasma, our approach involved the use of ultrafiltration for protein removal, covalent solid-phase extraction of the analytes of interest from the resulting ultrafiltrate with a phenyl boronic acid cartridge column, and analysis of the PBA eluant by narrow-bore reversed-phase HPLC.

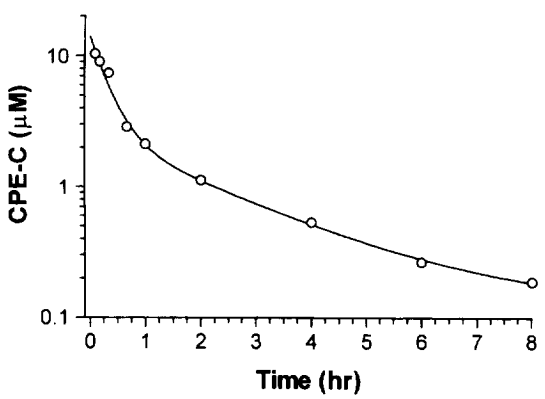

Fig. 4. Plasma concentration versus time curve for Sprague–Dawley rats given a 2 mg/kg i.v. dose of CPE-C. All time points represent the average concentration in two animals. The plotted curve represents the best non-linear least squares fit for a two-compartment open model for CPE-C plasma elimination.

4.1. Chromatography

Reversed-phase HPLC with UV detection has been employed extensively for the separation and quantification of nucleoside mixtures [19]. In general, pyrimidine nucleosides are less well-retained on reversed-phase columns than their purine analogues, and substantial attention must be paid to chromatographic conditions to achieve adequate resolution [20]. This was especially true for CPE-C, since cytidine analogues are generally the least lipophilic and the least retained of the pyrimidine nucleosides [21]. Adequate separation of CPE-C from endogenous pseudouridine and of isocarbodine internal standard from plasma uridine required a totally aqueous mobile phase, a C_{18} reversed-phase column with high carbon loading and well-deactivated silica, plus column conditioning and equilibration before use (Fig. 2A). This last requirement involved both an initial conditioning starting with 100% methanol and maintenance of column flow once equilibrium was established with 100% buffer. Retention and resolution of the nucleoside analytes were greater after this procedure than after equilibration with only the aqueous mobile phase. A similar effect with reversed-phase columns and water–methanol mobile phases was noted by Engelhardt et al. [22]. This effect can probably be attributed to the differential solvation of the hydrocarbonaceous bonded phase with residual methanol as the percent organic modifier decreases and a lessening of the self-aggregation and collapse of the alkyl chains, that has been postulated for totally aqueous mobile phases [23,24]. It should also be noted that any interruption of column flow caused a loss of nucleoside separation, presumably through disruption of this apparent dynamic equilibrium.

The potential requirement to carry out multiple analyses on a single, small volume, biological sample meant that the highest sensitivity possible be achieved while using a concentration sensitive detection method such as UV absorption. A narrow-bore (2 mm I.D.) column was selected as the best compromise between the high sample dispersion of an analytical (4 mm I.D.) column and the stringent dead volume and solvent delivery considerations required when using microbore (1 mm I.D.) columns [25]. At the optimum narrow-bore flow-rate of 0.150 ml/min, an adequate sample volume (10 μl) could be injected and the standard 8-μl flow cell of the UV detector could be used without noticeable loss of resolution.

A column switching system was employed to eliminate late-eluting components, such as purine nucleosides endogenous to plasma (Table 1), from the analytical column. A cartridge precolumn, which consisted of two narrow-bore guard column cartridges which could be easily and rapidly replaced, was connected to the analytical column through a 6-port, 2-position switching valve (Fig. 2). This electronically actuated switching valve was configured so that the precolumn could be backflushed to remove any components that were not transferred to the analytical column after a predetermined time. In practice, compounds with a $k'>5$ (Table 1) under the isocratic conditions employed were excluded from the analytical column. This allowed the precolumn to be thoroughly backflushed in preparation for the next analysis and permitted the use of isocratic conditions with a totally aqueous mobile phase for complete elution of all components from the analytical column in a reasonable time.

4.2. Sample preparation

The sample isolation scheme was designed based on the chemical properties of the analytes and the requirements of the analysis. All compounds of interest were pyrimidine nucleosides or analogues thereof, and all contained a *cis*-diol moiety (Fig. 1). These compounds also tended to be quite hydrophilic [21] and, in the case of CPE-C, did not bind to plasma proteins to any measurable extent. Thus centrifugal ultrafiltration using a membrane with a molecular mass cut-off of 30 000 was a convenient and efficient means of removing protein from plasma samples. A phenylboronic acid cartridge, in an activated state at pH 10, was then used to remove the *cis*-diols of interest from the plasma ultrafiltrate matrix. Although CPE-C was somewhat unstable upon prolonged exposure to basic pH, the 2 min or so required for loading the sample on the activated PBA cartridge was inconsequential. The activated PBA cartridge will covalently bind all *cis*-diols and α-aminoalcohols [26], so purine nucleosides and other plasma ultrafiltrate components will also be

retained. The cyclic boronate esters that were formed were decomposed and the analytes eluted under acidic conditions. The minimum volume possible of 2 *M* formic acid was used to elute these compounds from the PBA cartridge so that the original sample would not be further diluted. Likewise, solid NH_4HCO_3 was used to neutralize the formic acid eluate before chromatographic analysis for the same reason. Although the PBA cartridge does not have as large a sample capacity as an equivalent C_{18} solid-phase extraction cartridge or the same amount of ion-exchange resin, it can certainly accommodate a larger sample size than the 0.25 ml of ultrafiltrate that was used. Thus, if there are no constraints on the volume of the original sample, such as would be the case with samples of human origin, the PBA covalent solid-phase extraction step could also serve to concentrate the sample.

4.3. Assay characteristics

Isocarbodine (Fig. 1) was chosen as an internal standard on the basis of structural and chemical considerations. This saturated carbocyclic nucleoside is produced by the controlled stereospecific reduction of CPE-C [11] and is not a natural metabolite of CPE-C nor an endogenous constituent of plasma. Since isocarbodine contains a *cis*-diol moiety, it can be selectively isolated from plasma by covalent solid-phase extraction with a PBA cartridge in the same manner as CPE-C and with a similar recovery (Table 3). The cytosine base also gives isocarbodine the same chromophore as CPE-C and similar properties for UV detection (Table 1). In the chromatographic system employed for separation of CPE-C from endogenous pyrimidine nucleosides in plasma, isocarbodine has a clear chromatographic retention window (Fig. 3B) and has the potential to be used as an internal standard for the determination of pseudouridine, cytidine, CPE-U and uridine.

Linear calibration curves could be generated for CPE-C over the range 0.2–10 μM in all the biological matrices examined. In the rat, this made the detection of CPE-C possible at up to 24 h after a single bolus dose (see Section 4.4). For samples where the concentration of CPE-C was high, such as plasma obtained immediately after bolus dosing or the initial urine collection, dilution could be employed to bring CPE-C levels within the range of the calibration curve. The amount of isocarbodine added to sample aliquots was based on the estimated mid-range value of the expected of CPE-C concentrations. Since a calibration curve typically covered two-orders of magnitude of CPE-C concentration, this meant that area ratios fell between 0.1 and 10 for optimum precision. For rat plasma, which was the matrix most thoroughly investigated since rats were initially used for preclinical pharmacology studies [27], this assay had a within day precision and accuracy of better than 4% and 6%, respectively, for the range 1–20 μM CPE-C (Table 2).

4.4. Rat pharmacokinetics

The concentration versus time profile of Fig. 4 indicates the HPLC assay described here is well suited for pharmacokinetic studies. Following a single 2 mg/kg bolus dose of drug, CPE-C plasma levels were maintained in the range of in vitro antitumor activity for more than 8 h. Although a two-compartment model for plasma elimination was the most reasonable for the data obtained in this study, detectable levels of CPE-C at 24 h after dosing suggested a third phase of elimination with a much longer half-life. The urinary excretion data also implied an extended phase of elimination, since CPE-C could be measured in the 24 to 48-h urine collection. Subsequent pharmacology studies have allowed definition of this third phase of plasma elimination in mice, rats and dogs [27].

In rats, the majority of the CPE-C dose was excreted unchanged in urine. The deamination product of CPE-C, cyclopentenyl uridine (CPE-U), was not detectable in plasma nor was it measurable in urine. This was not unreasonable since CPE-C is a very poor substrate for cytidine deaminase and levels of this enzyme are lower in rats than in other species [10]. CPE-U has been detected and measured in rhesus monkeys [28,29] and humans [30], where it is a major metabolite, using the methodology described here. Assay characteristics for CPE-U were the same as those of CPE-C except that it was longer retained (Table 1) and its UV absorbance was monitored at 265 nm for maximum sensitivity, giving an LOQ of 0.3 μM [30].

5. Conclusion

A comprehensive narrow-bore HPLC method has been developed and evaluated for the measurement of CPE-C in biological samples at therapeutically relevant concentrations. This method has been applied to determine CPE-C plasma pharmacokinetics, catabolism to CPE-U and urinary disposition in rodents, dogs and non-human primates as part of the preclinical development of this new antitumor agent [10,28,29]. The described method has also been employed to define human pharmacokinetics and disposition during a Phase I clinical trial of CPE-C administered as a 24-h continuous infusion [30]. The current method sensitivity and the automation of the chromatographic analysis make this assay well suited for use in the further therapeutic development of CPE-C. Furthermore, the potential ability of this assay to measure simultaneously the CPE-C biomodulators cytidine and uridine as well as the tumor biomarker pseudouridine [31], make this method a useful tool to study other chemotherapeutic agents that affect pyrimidine biosynthesis.

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