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The cytotoxic drug cyclo-pentenyl cytosine: from manufacturing to anti-tumor activity and (cardio)toxicity

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CHAPTER 4

QUANTITATIVE ANALYSIS OF THE EXPERIMENTAL CYTOTOXIC DRUG CYCLOPENTENYL CYTOSINE (CPEC) AND ITS METABOLITE IN PLASMA WITH HPLC TANDEM MASS SPECTROMETRY

Submitted

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ABSTRACT

The cytotoxic drug cyclopentenyl cytosine (CPEC) is currently being investigated in early clinical trials. Monitoring of plasma levels is required for pharmacokinetic analysis and management of toxicity. This paper describes the analysis of CPEC and CPEU in plasma by HPLC-electrospray ionization tandem mass spectrometry. The calibration curves for CPEC and the metabolite cyclopentenyl uracil (CPEU) were linear up to 10 μM with correlation coefficients of 0.997 (SD = 0.002, $n = 10$) and 0.997 (SD = 0.004, $n = 10$), respectively. The detection limit for CPEC was 0.03 μM and 0.15 μM for CPEU. The intra- and interassay coefficients of variation for CPEC and CPEU were less than 10%. The usefulness of the method was demonstrated by analyzing CPEC and CPEU in plasma of a patient treated with CPEC. HPLC with electrospray ionization tandem mass spectrometry allowed rapid and sensitive determination of CPEC and CPEU levels in plasma.

INTRODUCTION

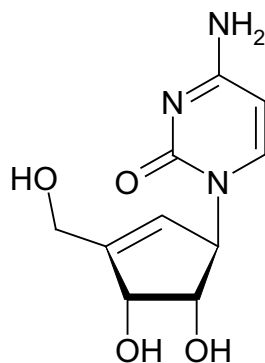
The experimental drug cyclopentenyl cytosine (CPEC) is a pyrimidine analogue of cytidine (figure 1). The active metabolite CPEC-triphosphate (CPEC-TP) is formed after transmembrane transport and the successive phosphorylation by the enzymes uridine-cytidine kinase, nucleoside monophosphate kinase and nucleoside diphosphate kinase. CPEC-TP inhibits cytidine triphosphate synthetase (CTP synthetase) which is known to catalyse the formation of cytidine triphosphate (CTP) from uridine triphosphate (UTP). Inhibition of CTP synthetase leads to diminished RNA and DNA synthesis and accumulation of cells in the S-phase of the cell cycle. Although CTP can also be synthesized by the salvage of cytidine, it has been shown that several malignant tissues predominantly use the pathway involving CTP synthetase [1,2]. CPEC can be deaminated to its metabolite cyclopentenyl uracil (CPEU). Pharmacokinetic data from animals revealed major interspecies differences. In rodents and dogs renal excretion of the unchanged drug was the primary route of elimination whereas in primates deamination to CPEU was dominant [3,4]. Although deamination to CPEU accounted for a significant part of the elimination in humans, elimination of CPEC occurred mainly by renal excretion of the unchanged drug [5].

CPEC has been studied in humans in a phase I trial involving 26 adults with solid tumors [5]. Hematological toxicity was dose limiting, however, the most serious adverse effect was severe hypotension. Five patients experienced in total 6 episodes of hypotension, which resulted in the death of two patients. As the events occurred in the higher dose groups, a dose-effect relationship was suggested. Animal studies gave no further indication of the underlying mechanism of this adverse effect [6]. Currently, we are initiating phase I/II trials with low dose CPEC in adult and pediatric patients with refractory hematological malignancies (acute myeloid leukemia, acute lymphoid leukemia and myelodysplastic disorders) under strict cardiac monitoring.

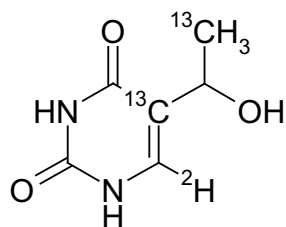
In the previous phase I trial, CPEC was measured in serum samples using a HPLC method with UV detection [7]. However, this method had a detection limit of 0.1 μ M for CPEC, and was expected not to be sensitive enough when using low dosage regimens. Moreover, the method was laborious requiring ultra filtration and subsequent solid phase extraction of the plasma before it could be subjected to HPLC analysis.

Therefore, we have developed a rapid, specific and more sensitive method, using HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI MS/MS).

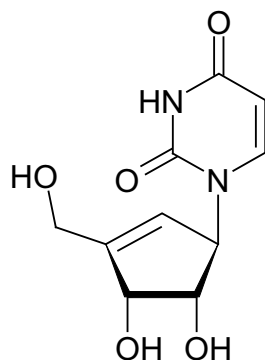
CPEC



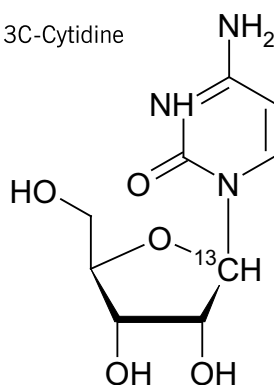
5-OH-Me-Uracil



CPEU



Ribose-1-13C-Cytidine

**Figure 1**

Chemical structure of CPEC, CPEU, 5-OH-Me-Uracil (+4) (internal standard for CPEC) and ribose-1-¹³C-Cytidine (internal standard for CPEU)

MATERIALS AND METHODS

Chemicals

CPEC (NSC375575) and CPEU (NSC375574) were kindly donated by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment of the National Cancer Institute (NCI, Bethesda, Maryland, USA). The internal standards 4,5-¹³C₂-5',5'-D₂-5-Hydroxymethyluracil (5-OH-Me-Uracil(+4)) and ribose-1-¹³C-Cytidine (Cytidine(+1)) were obtained from Cambridge Isotope Laboratories (Andover, Massachusetts, USA). Methanol, acetic acid and ammonia were obtained from Merck (Amsterdam, The Netherlands) and were of analytical or HPLC grade. Demineralised water from a Milli Q water purification system (Millipore, Amsterdam, The Netherlands) was used for preparation of stock solutions. Pooled plasma (free from CPEC and CPEU) from different individuals was obtained from the hospital laboratory.

Sample preparation

INTERNAL STANDARD

Stock solutions of Cytidine(+1), (99% pure, internal standard for CPEU) and 5-OH-Me-Uracil(+4), (99% pure, internal standard for CPEC) were prepared in demineralised water. A 1 mM solution of both stable isotopes was used as internal standard.

SAMPLES PREPARATION

Stock solutions of CPEC and CPEU consisted of adequately weighed quantities of CPEC and CPEU dissolved in purified water to obtain concentrations of 4.07 mM CPEC and 1.89 mM CPEU. These stock solutions were combined and further diluted in purified water to obtain working solutions of 400 μ M CPEC and CPEU. The working solutions were used to spike the plasma in appropriate concentrations. All solutions were stored at -20°C. For calibration and validation, concentration series of CPEC and CPEU in pooled plasma (0.03 – 10 μ M) were prepared.

A volume of 20 μ l internal standard was added to 200 μ l plasma. Proteins were precipitated by adding 10 μ l 11.6 M HClO₄ followed by centrifugation (5 minutes 10.000 x g). Subsequently 150 μ l of the supernatant was neutralized with 5 μ l 5 M K₂CO₃. After 10 minutes the samples were centrifuged in a microcentrifuge (5 minutes 10.000 x g) and an aliquot of 100 μ l of the supernatant was injected in the HPLC-MS/MS.

HPLC-electrospray ionization ms/ms

Separation of the components in the samples was performed on a Phenomenex Aqua C18 analytical column (250 x 4.6 mm) (Phenomenex, Torrance, USA). Solvent A consisted of 0.05 M acetic acid in water (pH set at 4.70 with 25% ammonia) and solvent B of methanol. Elution was performed by use of a gradient, at a flow rate of 1 ml/min, as follows: 0-6 min, 100% solvent A to 30% solvent B; 6-6.1 min, 30% solvent B to 80% solvent B; 6.1-9.0 min, 80% solvent B; 9.0-9.1 min, 80% solvent B to 100% solvent A; 9.1-15 min 100% solvent A. A splitter between the HPLC column and the mass spectrometer was used to introduce the eluate between 6.0 and 9.0 minutes at a flow of 50 μ l/min into the mass spectrometer. Samples were analyzed on a Waters Micromass Quattro II tandem mass spectrometer (Micromass, Manchester, UK) in the positive ion mode using electrospray ionization (ESI). Nitrogen was used as nebulizing gas and Argon as collision gas (collision cell pressure 2.5×10^{-3} mBar). The source temperature was set at 80°C and the capillary voltage at 3.5kV. Detection took place by multiple reaction monitoring (MRM) with a dwell time of 0.1 second per channel. The following transitions and settings were used: CPEC m/z 240>112 (cone voltage 35V); 5-OH-Me-Ura(+4): 147>86 (cone voltage 27V); CPEU 241>113 (cone voltage 35V), Cytidine(+1) 245>112 (cone voltage 28V). A collision energy of 15eV was used.

Validation

Linearity of CPEC and CPEU was tested by injecting calibration mixtures with different concentrations (10, 8, 6, 2, 0.5, 0.05 and 0.03 μM). The calibration curves were obtained from a least-squares regression for the analyte/IS peak-area ratio versus the concentration of the analyte in the calibration mixture. The correlation coefficients of the calibration curves for CPEC and CPEU were required to be ≥ 0.990 . The detection limit was established by injection of the calibration samples and defined as a signal to noise ratio of 3.

Intra-assay variation (intra-day precision) and the accuracy of the method was established by measuring plasma enriched with CPEC and CPEU at low (0.1 μM for CPEC and 0.5 μM for CPEU), medium (1 μM) and high (4 μM) concentrations (10 samples of each concentration in a single run). The inter-assay variation (inter-day precision) and accuracy was established by measuring the low, medium and high concentrations and the calibration line on 10 different days. The concentration of each sample was calculated using the calibration curves. The coefficients of variation were considered acceptable in case they were below 10%. Accuracy was expressed as [(overall mean concentration/nominal concentration) $\times$ 100%], and should be within the range from 85 to 115%. Three samples with 0.1 μM CPEC or 1 μM CPEU were analyzed at different time points after storage at -20°C (0, 1 and 2 months).

The influence of the matrix was tested by determination of the recovery of CPEC and CPEU in plasma samples of 10 different patients spiked with medium and low concentrations of CPEC and CPEU.

Suitability of the method was tested by analyzing plasma samples that were obtained at different time points from a patient treated with CPEC in a phase I/II trial (approved by the Medical Ethical Committee of the Academic Medical Center) at our institute. In addition plasma from a mouse that was treated with CPEC for an *in vivo* study (approved by the Ethical Animal Research Committee of the Leiden University Medical Center) was also analyzed.

RESULTS

The calibration curve for CPEC was best described by a weighted (1/x) quadratic curve ($R^2 = 0.997$, $SD = 0.002$, $n = 10$) and not by a linear curve as non-linear responses at the higher points of the validation curve were observed. The calibration curve for CPEU was linear in the concentration range tested ($r^2 = 0.997$, $SD = 0.004$, $n = 10$). The detection limit (defined as a signal-to-noise ratio of 3) for CPEC proved to be $0.03 \mu\text{M}$ and for CPEU $0.12 \mu\text{M}$. As the detection limit of CPEU was below the lowest validation point, we analyzed a spiked plasma sample with a concentration of $0.15 \mu\text{M}$ ($n=3$) and found an accuracy of 90%. Therefore the detection limit of CPEU was set at $0.15 \mu\text{M}$. After storage at -20°C we found an accuracy of 92% (mean 0.09 , $SD 0.006$) for CPEC and 103% for CPEU (mean 1.03 $SD 0.06$).

Representative chromatograms of the analysis of a plasma sample spiked with CPEC ($0.1 \mu\text{M}$) and CPEU ($0.5 \mu\text{M}$) are shown in figure 2.

The intra- and inter-assay variations and accuracy for the different concentrations analyzed are represented in tables 1 (CPEC) and 2 (CPEU). The coefficients of variation (CV) were all below 10%. The recovery of CPEC and CPEU in the different patient samples is presented in table 3.

Table 1 Intra- and inter-assay variation and accuracy for CPEC

	Intra-assay			Inter-assay		
	CV, %	Mean (SD)	Accuracy (%)	CV, %	Mean (SD)	Accuracy (%)
$0.1 \mu\text{M}$	7.5	0.1 (0.007)	92	6.2	0.1 (0.009)	107
$1 \mu\text{M}$	5.9	1.0 (0.06)	103	5.08	1.1 (0.05)	109
$4 \mu\text{M}$	1.3	4.1 (0.05)	103	6.6	3.9 (0.3)	98

Table 2 Intra- and inter-assay variation and accuracy for CPEU

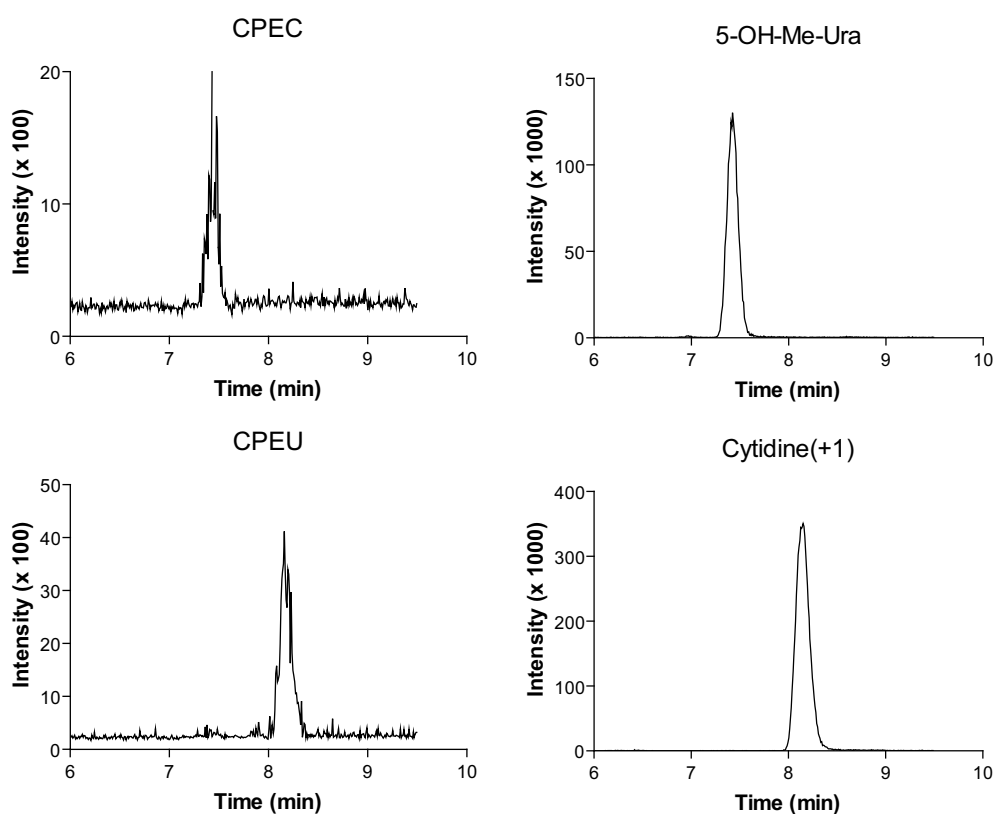
	Intra-assay			Inter-assay		
	CV, %	Mean (SD)	Accuracy (%)	CV, %	Mean (SD)	Accuracy (%)
$0.5 \mu\text{M}$	3.9	0.5 (0.02)	97	3.7	0.5 (0.02)	102
$1 \mu\text{M}$	4.4	1.0 (0.04)	97	5	1.1 (0.05)	106
$4 \mu\text{M}$	4.1	3.7 (0.2)	93	8.5	4.0 (0.3)	100

Table 3 Recovery in 10 different plasma samples

	CPEC		CPEU	
	CV, %	Recovery, % (SD)	CV, %	Recovery % (SD)
0.1 μ M	10.1	100.0 (1.0)	nd	nd
1 μ M	10.0	99.0 (1.0)	4.5	95.0 (4.2)

nd = not determined

In plasma samples from a patient treated with low dose CPEC (1 mg/m²/h for 24 hours), CPEU could be detected at the end of the infusion period and CPEC could be detected up to 48 hours after the end of the infusion (figure 3). When analyzing the plasma of a mouse treated with CPEC (5 mg/kg on 3 consecutive days) a concentration of 0.24 μ M for CPEC was found, whereas no CPEU could be detected in the plasma of a control mouse that was treated with saline.

**Figure 2**

HPLC-ESI MS/MS chromatograms of CPEC and internal standard cytidine(+1) and CPEU and internal standard 5-OH-Me-Uracil(+4).

DISCUSSION

The first Phase I study with CPEC performed by Politi *et al*, showed that plasma levels of CPEC were in the low micromolar range. That study described two rapid phases of elimination after a bolus injection (24 mg/m²) in two patients. A late phase of elimination was also suggested, however, it was not possible to describe this phase of the pharmacokinetics profile as plasma concentrations 24 h after the end of the infusion were below the limit of quantitation of the used HPLC-UV method. Plasma levels in that study increased linearly with CPEC dose. The phase I study was terminated because of severe cardiovascular adverse events in the patients receiving more than 2.5 mg/m² CPEC [5]. Due to these adverse effects, new trials with CPEC will apply lower dosages (0.5-1.5 mg/m²/h for 24 hours) and remain under 2.5 mg/m²/h. Monitoring of the plasma levels will be necessary to detect possible toxic plasma levels and to further explore CPEC pharmacokinetics. With lower dosages of CPEC, plasma levels are expected to be lower than 0.1 μ M shortly after the end of the infusion. In order to be able to measure these low plasma CPEC concentrations, the use of a sensitive method allowing the quantification of CPEC at concentrations below 0.1 μ M CPEC is necessary. Therefore, we developed a new HPLC-ESI MS/MS method, which proved to have the required sensitivity. With the method described and our instrumentation, CPEC can be detected at concentrations as low as 0.03 μ M and CPEU at 0.15 μ M. An additional advantage is the high specificity of the method making extraction unnecessary and offering an easy and rapid workup of the plasma samples. We found no signs of degradation of CPEC and CPEU upon storage. Earlier experiments also showed no degradation of CPEC for several years in aqueous solutions [8] and CPEC was found to be enzymatically and hydrolytically stable in plasma [7].

Internal standards for CPEC and CPEU were added to the plasma samples to compensate for losses during sample preparation and sensitivity loss due to quenching of the signal in the mass spectrometer. Cytidine(+1) and 5-OH-ME-Uracil(+4) were chosen as no isotopically labelled CPEC and CPEU were available.

In the phase I study the maximum concentration of CPEC after a bolus injection of 24 mg was approximately 10 μ M. This was also the maximum concentration in our calibration line. Steady state plasma concentrations in the study increased from 0.4 to 3.1 μ M at dose levels of 1 to 5.9 mg/m²/h. As in the phase I/II study in our institute dose levels of 0.5-1.5 mg/m²/h CPEC would be applied, we expected lower steady state plasma concentrations and therefore the lowest concentration for testing the intra- and inter-assay variation was 0.1 μ M. The samples of the patient treated with low dose CPEC confirmed that plasma levels remain in the lower range as a maximum concentration of 0.58 μ M of CPEC was detected. CPEU levels were lower than CPEC levels and at several time points the concentrations were below the detection limit of 0.15 μ M. However, after the end of the infusion a maximum concentration of 0.16 μ M was found. The low CPEU concentrations are in line with previous data from the phase I study indicating that

deamination of CPEC is not the primary route of excretion in humans [5]. CPEU has almost no cytotoxic effects [9,10]. Moreover, cardiotoxicity in the phase I study was not associated with CPEU concentrations. Therefore, the inability to quantify very low CPEU concentrations will not hamper the use of our method in clinical practice.

As demonstrated by the plasma levels of CPEC of the mouse, the method is also suitable for use in animal studies. Moreover, analysis of the plasma of an untreated mouse, suggested no interference of the difference in matrix with the method.

In conclusion, with the described HPLC-ESI MS/MS method we show that plasma concentrations of CPEC and CPEU can be measured at low concentrations with a simple and rapid preparation of the samples. The method can be used for monitoring plasma levels of patients treated with CPEC.

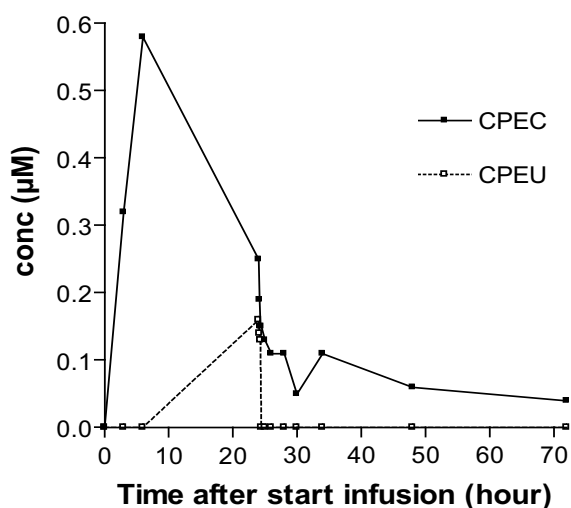


Figure 3

CPEC and CPEU plasma concentrations of a patient treated with CPEC (1 mg/m²/h for 24 hours).

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