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

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CHAPTER 3

FORMULATION, QUALITY CONTROL AND SHELF LIFE OF THE EXPERIMENTAL CYTOSTATIC DRUG CYCLOPENTENYL CYTOSINE

Drug Development and Industrial Pharmacy 2006;32:497-503

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ABSTRACT

This paper describes the formulation and quality control of an aqueous sterilized formulation of the experimental cytostatic drug cyclopentenyl cytosine (CPEC) to be used in Phase I/II clinical trials. The raw drug substance was extensively tested. A High Performance Liquid Chromotography (HPLC) method was validated for the quality control of the formulated product. The aqueous formulation was found to be stable for at least 2 years at 2-8ºC. Sterilization (15 min at 121 ºC) showed no influence on drug stability. The results show that CPEC can be formulated in an aqueous solution. The described HPLC method is a useful tool in the pharmaceutical quality control.

Keywords: Cyclopentenyl cytosine, CPEC, Cytostatic drug, Formulation, Quality control

INTRODUCTION

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC, Fig. 1) is a pyrimidine analogue of cytidine, currently entering phase I/II trials in recurrent leukemia. After transmembrane transport, CPEC is subsequently activated by phosphorylation by the enzymes uridine-cytidine kinase, nucleoside monophosphate-kinase (NMP-kinase), and nucleoside diphosphate-kinase (NDP-kinase) respectively to form CPEC-triphosphate (CPEC-TP), the major intracellular pharmacologically active metabolite. Cyclopentenyl cytosine-triphosphate (CPEC-TP) is an inhibitor of cytidine triphosphate-synthetase (CTP-synthetase) resulting in inhibition of RNA and DNA synthesis and leading to S-phase accumulation (Fig. 2). So far, CPEC pharmacokinetics and toxicity have been studied in a single phase I trial in 26 patients with solid tumors (in majority colorectal carcinoma). Dose limiting toxicity was of hematological nature. However, the most severe adverse drug reaction was cardiovascular: six episodes of hypotension occurred in five patients (dose range: 3-4.7 mg/m^2/h) and two patients, treated with the highest applied dose (4.7 mg/m^2/h), had a fatal hypotensic episode [1]. In the early phase I study, a lyophilized formulation of CPEC dispensed by the National Cancer Institute (NCI, Bethesda, Maryland, USA), was applied. In the present article we describe the development of an aqueous drug formulation of CPEC for intravenous use in our phase II trial and methods for the pharmaceutical quality control of the raw drug substance and formulated product.

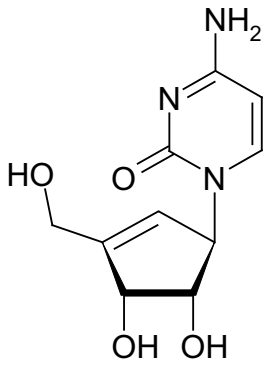

Figure 1 Chemical Structure of CPEC.

The mechanism of the cardiotoxic effects remains yet unclear [2] and has hampered the initiation of phase II studies with CPEC. In various human and animal leukemia models, antitumor activity of CPEC was demonstrated at relatively low drug concentrations [3,4]. Following these observations, we recently have initiated a phase II study of low dose CPEC in adults with hematological malignancies (acute myeloid leukemia, acute lymphocytic leukemia, and myelodysplastic syndrome) under strict cardiac monitoring. Furthermore, a phase I/II trial in pediatric patients with recurrent leukemia is planned.

MATERIAL AND METHODS

Chemicals

Cyclopentenyl cytosine (CPEC) (NSC375575, lotnr. BK-09-142) was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment of the NCI. For the manufacturing, 10 ml glass vials (DIN-I, Aluglas, Uithoorn, Netherlands) were used to contain the product solution and sterile 0.2 μm cellulose acetate filters (Millipore, The Netherlands) for filtration. Water for injections was manufactured in house (conform PhEur) (Department of Pharmacy, Academic Medical Centre, Amsterdam, The Netherlands).

The following analytical grade chemicals were used for quality control: methanol (Labscan Ltd, Ireland); ammoniumacetate (Merck, Darmstadt, Germany), 5- methylcytosine (Sigma-Aldrich, Munich, Germany), and tetrahydrofuran (THF, Merck, Darmstadt, Germany).

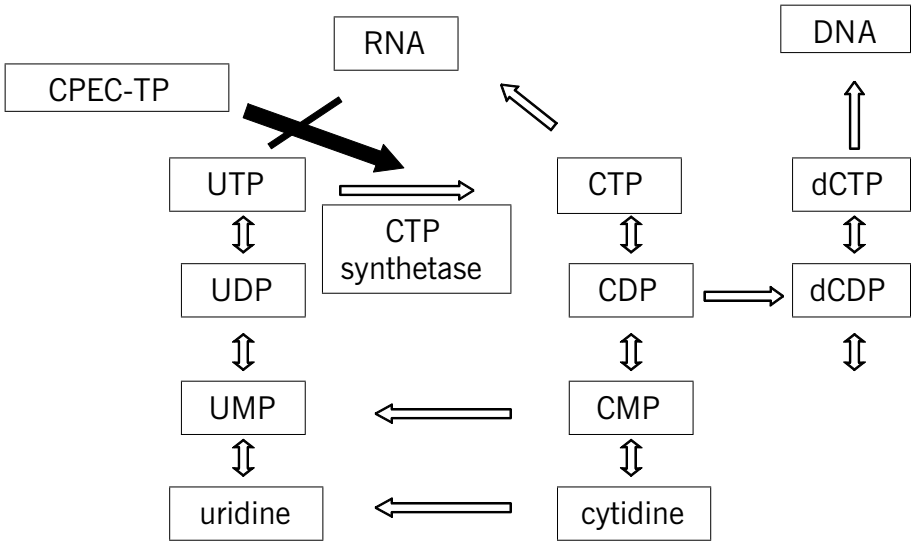

Figure 2 Mechanism of Action of CPEC.

In the figure a part of the pyrimidine (deoxy) ribonucleotide synthesis is shown. The enzyme CTP-synthetase is inhibited by CPEC-TP, leading to depletion of the CTP pool and subsequently inhibtion of DNA and RNA synthesis. CPEC-TP: CPEC-triphosphate; UTP: Uridine-triphosphate; UDP: Uridine-diphosphate; UMP: Uridine-monophosphate; CTP: Cytidine-triphosphate; CDP: Cytidine-diphosphate; CMP: Cytidine-monophosphate; dCDP: Deoxycytidine-diphosphate; dCTP: Deoxycytidine-triphosphate.

Quality Control of Drug Substance

The raw active substance was stored at 20ºC in a glass airtight container. The National Cancer Institute (NCI) initially performed identity and purity tests on each lot of the bulk drug substance (personal communication, Dr. R. Vishnuvajjala). The general tests performed by the NCI consisted of appearance, melting point, optical rotation, and elemental analysis. Conformity was further tested with UV (ultra violet), IR (infrared), MS (mass spectrometry), H-1, and C-14 NMR (nuclear magnetic resonance). Furthermore, the drug substance was chromatographically tested [thin layer and reverse phase high performance liquid chromatography (RP-HPLC)].

Before drug formulation, the following quality control tests on the raw material were performed (Laboratory of Clinical Pharmacy & Toxicology, AMC, Amsterdam).

IDENTITY

Identity was confirmed by infrared spectroscopy (FTIR-8201PC Shimadzu Corp., Japan), LC-MSMS product ion scan, concentration app. 100 μg/ml and UV-VIS spectroscopy (UV-2410 PC, Shimadzu, 8.4 mg/ml in H2O).

LOSS ON DRYING

An amount of approximately 100 mg CPEC (precisely weighed) was dried for 4 h at 100-105ºC in a pre-dried glass vial (1 h at 100-105ºC).

HEAVY METALS

Presence of heavy metals was not tested as no heavy metals that can be detected by the USP method were used in the synthesis of CPEC.

QUALITATIVE DRUG ANALYSIS

Analysis of impurities of the drug substance and content of the product was performed using an RP-HPLC equipped with a UV photo diode array detector (Jasco MD 1510 Multiwavelength detector, Jasco Corp., Japan), an autosampler (Jasco AS 1555 Intelligent sampler), and a pump (Jasco PU 1580 HPLC pump). Chromatograms were processed using Empower software (Waters, Netherlands). Separation was achieved using a Supelcosil LC-18 column (25 cm x 4,6 mm, 5 μm) (Supelco, Sigma-Aldrich, Netherlands). The method was originally developed at the NCI. The mobile phase consisted of 2% methanol in 0.1 M ammoniumacetate at a flow rate of 1.0 ml/min. The detection wavelength was 276 nm. The injection volume was 20 μl and a run time of 20 min was employed. A concentration of 24 mg CPEC/l and 50 mg CPEC/l was used for analysis of impurities of the drug substance. A concentration of 4.5 mg/l was used for the analysis of content of the product. The internal standard used was 5-methylcytosine (5 mg/l).

Validation of HPLC Procedure

Validation of the method of analysis was performed according to good clinical laboratory practice (GCLP) guidelines as follows.

DETERMINATION OF THE SELECTIVITY AND SPECIFICITY

Impurities or degradation products were required to elute separately from CPEC in order to assess possible impurities. Cytosine is used in the synthesis of CPEC, and dideoxycytidine and cytarabine have a strong structural relationship with CPEC. Therefore, cytosine, dideoxycytidine, and cytarabine were examined. In order to test if degradation products could be detected and separated, samples of CPEC were exposed to extreme temperature (48 h at 120ºC), acid and alkaline conditions, respectively. All substances were dissolved in water and injected onto the HPLC system. To evaluate proper separation from CPEC, the resolution factor (R) between CPEC and the nearest peak (internal standard, 5-methylcytosine) was determined. To obtain a separation of peaks of at least 99.7% for quantification, a resolution factor of at least 1.5 was required.

DETERMINATION OF RANGE

The final product (CPEC, 4.5 mg/ml) was diluted to obtain a concentration suitable for quantification with HPLC. A quantification range of 75% and 125% of this concentration was chosen.

ACCURACY AND RECOVERY

As the final product will be dissolved in an aqueous solution, which will only have to be diluted for analysis, no tests on accuracy and recovery were performed.

REPEATABILITY AND INTERMEDIARY PRECISION

Repeatability was tested by analyzing a reference sample within one day (n = 6) and on six different days. Repeatability was determined for four different concentrations (90%, 95%, 105%, and 110% of the nominal product concentration). Concentrations were calculated relative to the 100% value (“one point calibration”). The mean, standard deviation, and variation coefficient were determined of each series. Repeatability variation coefficients were considered acceptable below 5%. Intermediary precision was performed in the same way as the repeatability but with varying equipment, technician, and eluent lot.

LINEARITY

Reference samples with 75%, 90%, 95%, 100%, 105%, 110%, and 125% of the declared product concentrations were analyzed on six different days. A calibration line was calculated for each different day. Each reference sample was recalculated on the calibration line obtained with the other reference samples of that day. The obtained individual concentrations were not

allowed to differ more than 5% of the nominal values. Linearity ($y = ax + b$) was tested with the "goodness of fit test" (GOF-test) using SPSS software (version 9.0). An F-value of the test of at least 7.71 and an r^2 value of >0.990 were considered acceptable.

ROBUSTNESS AND SUITABILITY OF THE METHOD

In order to test whether the method could be used for the final (sterilized) product, two reference samples were sterilized (15 min at 121ºC) before analysis. The following changes in chromatography conditions were tested in order to investigate whether the separation could be further optimized: change of the MeOH (Methanol) concentration in the mobile phase (1% instead of 2%) and addition of THF.

Drug Formulation

Cyclopentenyl cytosine (CPEC) was dissolved in purified water at a concentration of 4.5 mg/ml (dry weight, determined one day before production). The dissolved product was filtered through a 0.2 μm membrane filter in a class A laminar flow safety cabinet with class D background environment. Ten milliliter sterile vials were filled with 2.0 ml of the product solution. Vials were sterilized (15 min 121ºC) in a heat water autoclave. As initially little information was available on the stability of CPEC in an aqueous solution, the vials were stored at 4ºC after sterilization.

QUALITY CONTROL OF PRODUCT

Before the product could be used, several analyses were performed. Labeling, volume, pH, and physical appearance had to comply with product specifications. Sterility was assured by parametric release. Content was assessed in duplo by using the above described HPLC method.

SHELF LIFE EXPERIMENTS

In order to test whether the content of the product remained stable until the end of the planned expiration date, two different batches of the product were analyzed on several time points up to 24 months after production.

RESULTS

Quality Control of Drug Substance

IDENTITY

Infrared spectroscopy showed a spectrum consistent with the chemical structure and with results obtained earlier (certificate of analysis, NCI). The UV-VIS spectrum showed a major absorption band with a maximum at 276 nm which corresponded with earlier data. The mass spectrum also complied with the chemical structure of CPEC (Fig. 3).

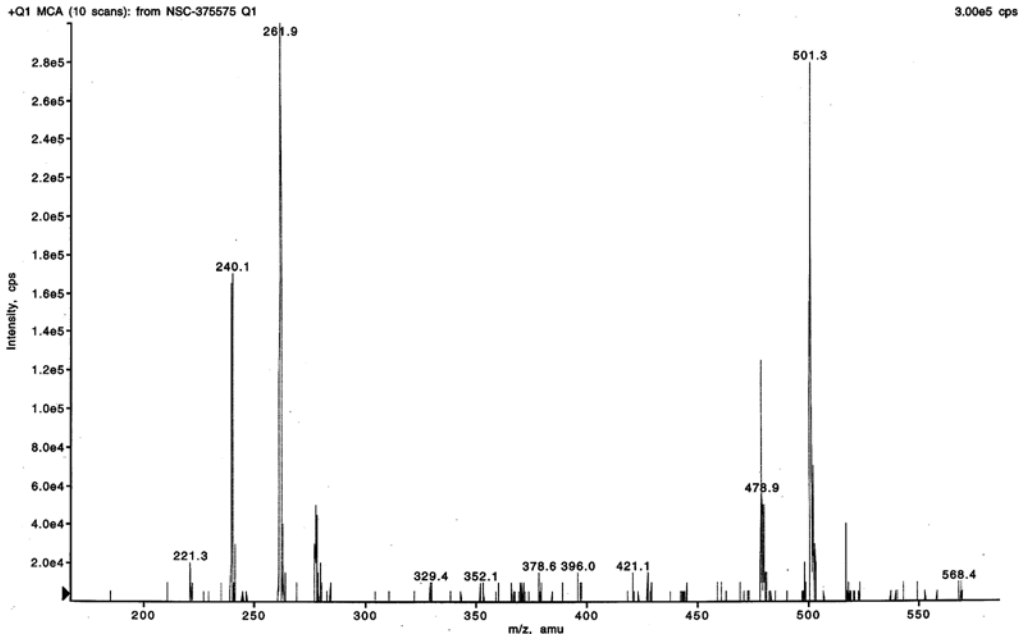

Figure 3 Mass Spectrum of CPEC

LOSS ON DRYING

Loss on drying was determined on two samples; the loss on drying was respectively 9.07% and 9.93%, with a mean loss of 9.5%.

Validation of the HPLC Method

DETERMINATION OF THE SELECTIVITY AND SPECIFICITY

Cyclopentenyl cytosine (CPEC), dideoxycytidine, 5-methylcytosine, and cytarabine eluated well separated from the column. The resolution between CPEC and the internal standard (5-methylcytosine) was 35 which is above the limit of acceptance of 1.5. A representative chromatogram of a reference sample containing CPEC and the internal standard is shown in Fig. 4. The chromatogram of the sample which was kept 48 h at 120ºC, showed a minor unidentified peak next to the CPEC peak, probably indicating a degradation product. The chromatograms of the samples submitted to acid and alkaline conditions were similar to the unexposed reference sample.

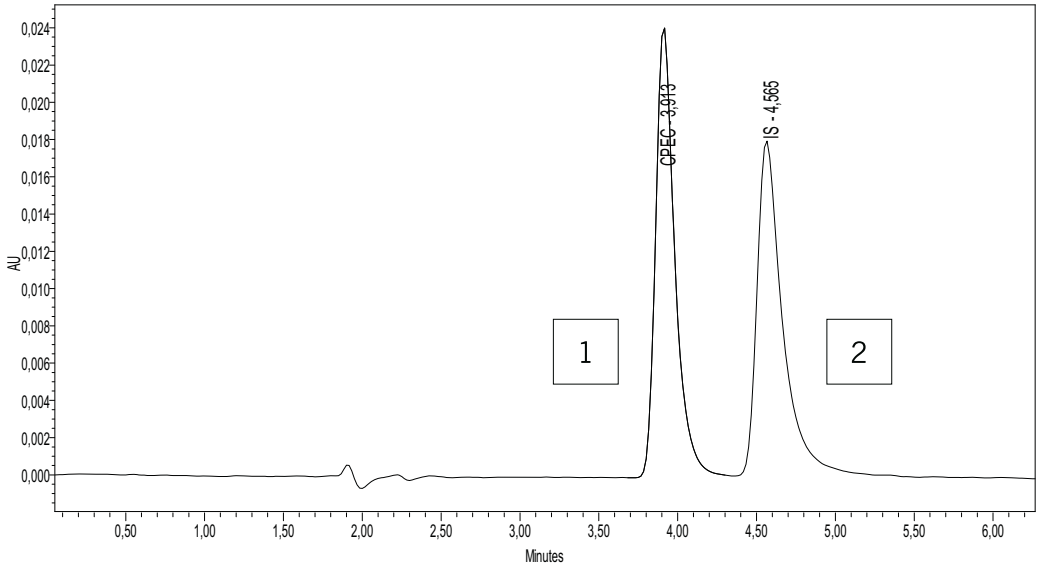

Figure 4 Characteristic HPLC Chromatogram of CPEC. 1: CPEC. (4.5 mg/l); 2: 5-methylcytosine (Internal Standard, 5 mg/l).

DETERMINATION OF RANGE

Dilutions of the final product to 75% (3.375 mg/l) and 125% (5.625 mg/l) of the declared product concentration could be measured and quantified.

REPEATABILITY AND INTERMEDIARY PRECISION

The repeatability and intermediary variation coefficients for all the determined concentrations (90%, 95%, 105%, and 110%) were less than 3%. Mean, standard deviation, and variation coefficients for the intra (six in one day) and inter (six different days) day repeatability are shown in Table 1.

Table 1 Repeatability and intermediary precision of HPLC method

	Mean concentration (%)		Standard deviation		Variation coefficient (%)	
90%	repeatability	intermediary	repeatability	intermediary	repeatability	intermediary
Within 1 day	91.07	89.50	0.58	2.04	0.63	2.28
6 days	90.37	91.41	0.91	1.02	1.01	1.12
95%						
Within 1 day	93.69	94.84	0.49	2.00	0.53	2.07
6 days	95.41	95.54	1.34	0.64	1.40	0.67
105%						
Within 1 day	104.82	105.10	2.12	1.21	2.03	1.14
6 days	103.99	105.66	1.32	1.09	1.27	1.03
110%						
Within 1 day	109.24	111.17	2.31	2.16	2.11	1.94
6 days	109.06	110.74	1.48	1.33	1.36	1.21

LINEARITY

All seven-point calibration curves of CPEC were linear with a mean correlation coefficient of 0.994 (standard deviation 0.004) and F-values above 7.71. The maximum difference of the individual concentrations from the nominal concentrations was 1.31% (see Table 1).

ROBUSTNESS AND SUITABILITY OF THE METHOD

The chromatograms of the sterilized product were not different from the non-sterilized standard solution. Changes in chromatographic condition did not further improve the separation (data not shown).

Formulation of Product

Two lots (batch size: 100 vials) were produced and could be released for clinical use after quality control. Shelf life experiments showed no deterioration in CPEC concentration (Table 2) at 24 months after production. The lowest concentration after two years in the two lots was 97.2 and 99.5%, respectively, of the declared concentration. After 18 months the concentration seemed to increase. A possible cause for the assumed increase could be analytical and probably due to insufficient drying of the standard used in the HPLC method. The results at 20 and 24 months seemed to confirm this assumption as the concentrations remained then within 10% of the declared value.

Table 2 Shelf life experiment

month s of storage	Content CPEC batch 1 (% of declared concentration	Content batch 2 (% of declared concentration, mean of two assays)
0	102.0 [101.66-102.30]	99.3 [99.3-99.3]
2	99.7 [99.6-99.7]	*
9	99.5 [99.0-99.9]	97.2 [96.4-98.0]
11	101.3 [101.32]**	102.4 [102.38]**
18	112.6 [112.2-113.0]	109.4 [109.2-109.6]
20	106.5 [106.1-107.0]	108.1 [107.7-108.4]
24	108.3 [106.2-111.5]	101.2 [98.1-104.3]

The percentages in the table represent the mean of two assays, between brackets the individual values are given.
*Not determined
**Only one assay performed.

DISCUSSION

In an early and single Phase I trial of CPEC in patients with solid tumors, a lyophilized formulation of CPEC was used. A drawback of this approach is that lyophilization facilities are required for drug formulation and once formulated the presentation form needs reconstitution before administration. However, in case of labile compounds, lyophilization permits much longer storage as compared to aqueous solutions. As pilot experiments suggested good stability of CPEC in aqueous solutions, we investigated whether the production of an aqueous CPEC drug formulation was feasible. We started our experiments with testing the raw drug substance. As no Pharmacopoeia monograph is available, quality control criteria were absent and a set of specifications and analytical methods were determined in our laboratory based upon information from the NCI. The chosen HPLC method was developed for pharmaceutical quality control and not for the determination of CPEC concentrations in biological specimens such as serum. This implicates that stability indicating performance and the precision at the declared product concentration were considered more relevant than e.g., the limit of detection. The method was validated and found to be precise, linear, and stability indicating. The HPLC method was accepted for use in pharmaceutical quality control of both the raw active substance and the product. The initial expiration date was set at three years after production and actual parent drug concentration was frequently monitored at a shelf life experiment during storage at 4ºC. Currently, after two years of shelf life, the CPEC concentration remained above 97% of the declared concentration. Measurements will be continued for the remaining storage time. Before administration the product can be further diluted with standard infusion solutions. There is no reason to assume that the product will be less stable after dilution. However, as the product does not contain preservatives, a short period of usage will be advised for microbiological reasons. This study shows that CPEC can be formulated in an aqueous solution and stored for at least two years at 4ºC. The described HPLC method is a useful tool in the pharmaceutical quality control of the drug substance and product.

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