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Short communication

Development and validation of a sensitive reversed-phase HPLC method to determine intracellular accumulation of hydroxycamptothecin

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Abstract

The intracellular accumulation of anti-cancer agents strongly influences the efficiency of chemotherapy for cancer. In the present study, a simple, rapid, sensitive reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated to determine hydroxycamptothecin (HCPT) in Eca109 cells. HCPT in cellular lysis solution were measured by RP-HPLC with a C_{18} column after extraction with ethyl acetate. The mobile phase contained 0.1% triethylamine–phosphoric acid buffer (pH 3.0) and acetonitrile (75:25, v/v). Fluorescence detector with excitation and emission wavelengths of 382 and 528 nm was used for determination of HCPT. The calibration curve was linear from 2 to 100 ng/ml with correlation coefficient of 0.9999, while the limit of quantification is 2 ng/ml. The recovery of assay was between 86.5 and 105.2%. The intra- and inter-day coefficients of variation were less than 10% (R.S.D.). Furthermore, the validated method was used to determine the accumulation of HCPT after incubating the liposomal formulation of HCPT and HCPT for injection with the intact cells. HCPT liposomes showed higher intracellular accumulation of HCPT at different incubation times compared with that of conventional HCPT injection.

Keywords: Hydroxycamptothecin; High-performance liquid chromatography; Cells; Determination; Liposomes

1. Introduction

Camptothecin (CPT) is a natural alkaloid isolated from extracts of camptotheca acuminata in 1958 [1]. As a kind of promising anti-tumor agent, camptothecin and its analogues have been paid considerable attention. In the 1990s, several CPT analogues were synthesized and isolated, including irinotecan, topotecan and 10-hydroxycamptothecin (HCPT) [2]. The anti-tumor mechanism of these compounds is based on the inhibition of DNA replication and RNA transcription by stabilizing the cleavable complexes formed between topoisomerase I and DNA [3].

HCPT exists in two distinguishable forms, an active α-hydroxy-δ-lactone ring and an inactive carboxylate form at different pH conditions. An acidic pH promotes the formation of the active lactone ring, while a more basic pH favors the inactive carboxylate formation. Crow and Crothers [4] and Wani et al. [5] indicated that a closed hydroxylactone ring moiety is an important structural requirement for successful drug interaction with the Topo I target and anti-tumor potency.

The above observations indicate that the anti-tumor activity of HCPT depends on the extent to which it associates with topoisomerase I and DNA and also on the existing form of drug. Some studies of in vitro cytotoxicity and intracellular drug uptake also indicated that increasing intracellular anti-cancer drug level was likely to lead to an enhanced cytotoxic potency [6–9]. Therefore, it is necessary to develop a method for the determination of intracellular accumulation of hydroxycamptothecin. Croce et al. [10] determined the intracellular accumulation of two camptothecin analogues (topotecan and gimatecan) using semi-quantitative approach of fluorescence intensity. Up to now, there is no effective quantitative analysis method for the determination of intracellular accumulation of hydroxycamptothecin.

On the other hand, liposome delivery systems may achieve controlled release or enable drug targeting to specific tumor sites and improve drug stability [11–13]. Chemotherapeutic agents via liposomes with efficient intracellular uptake and accumulation in an intracellular site of action should be the determinant

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step for their anti-cancer activity [14]. Here, we constructed an effective, specific, precise and sensitive RP-HPLC method to measure HCPT in Eca109 cells. The intracellular accumulation of HCPT in Eca109 cells incubated with either HCPT liposomes or HCPT injection was also evaluated using the validated method.

2. Experimental

2.1. Chemicals, reagents and cells

HCPT (purity >98.6%) was provided by China Aroma Chemical Co. (Hangzhou, China). HCPT injection were purchased from Harbin Sanctity Pharmaceutical Co. (Harbin, China). Acetonitrile (ACN) and methanol of HPLC grade were obtained from Siyou Chemical Reagent Co. (Tianjin, China). A human esophageal cancer cell line, Eca109, was obtained from Henan Key Laboratory of Molecular Medicine (Zhengzhou, China). Dulbecco's Modified Eagle Medium (DMES) and trypsin were purchased from Genom BioMed Technology Inc. (Hangzhou, China). Fetal bovine serum was purchased from Beijing Yuan Heng Sheng Ma BioMed Tech Inc. (Beijing, China). Cholesterol was purchased from Pharmacia Biotech (Piscataway, NJ, USA), and Soya phosphatidylcholine from Lipoid GmbH (Ludwigshafen, Germany). All other chemicals were of analytical grade from a variety of suppliers.

2.2. Cell line and culture conditions

The human Eca109 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum. The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, and were subcultured every 2 days with 0.25% trypsin.

2.3. Chromatographic conditions

The HPLC system used for drug content analysis consisted of Agilent 1100 modules (Wilmington, DE, USA), with a quaternary pump, mobile phase degasser, auto-sampler with thermostat and a column heater compartment. ChemStation was used for data acquisition and analysis. For HCPT assay, the chromatographic condition was set up as follows: an Agilent Zorbax SB-C_{18} guard column (4.6 mm × 12.5 mm, 5 μm); analytical column (4.6 mm × 150 mm, Diamonsil^{TM} C_{18}, 5 μm, purchased from Dikma Technologies Inc.); mobile phase, a 75:25 (v/v) mixture of 0.1% triethylamine–phosphoric acid buffer (pH 3.0) and ACN; flow-rate, 1.0 ml/min; degassed with helium on line, 30 ml/min; fluorescence detection, excitation 382 nm, emission 528 nm; injection volume, 20 μl; temperature 35 °C. The HCPT content in the formulation was determined using external standards.

2.4. Sample preparation

The cells were collected by centrifugation. The resulting pellets were resuspended in 1.0 ml phosphate buffered saline (PBS). Cells were lysed over ice by sonication (6/8 s, 200 W, 20 times). Fifty microliter phosphoric acid was added to 1.0 ml aliquots of cell lysis solution (10^7 cells/ml) for HCPT in the closed lactone ring form and vortexed for 3 min. The mixture was extracted with ethyl acetate, vortexed for 5 min, and centrifuged for 15 min at 3500 rpm. The upper layer was transferred into another test tube and evaporated to dryness with N_2 at 35 °C. The residue was reconstituted in 100 μl methanol, vortexed for 3 min and centrifuged for 10 min at 3500 rpm before analysis. Twenty microliter of the sample was injected into the HPLC system.

2.5. Calibration curves

To prepare calibration curves, standard samples of HCPT were added to blank cell lysis solution to give final concentrations of 2, 5, 10, 20, 40, 80 and 100 ng/ml. Calibration curves were constructed by performing a linear regression analysis of the peak area (y) versus the HCPT concentrations (x).

2.6. Validation of the method

The limit of detection (LOD) in standard solution was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) was defined by concentration with a signal-to-noise ratio of 10. The intra-day precision of the assays performed in replicate ($n = 5$) were tested by using three concentrations of the individual HCPT, 2, 20 and 80 ng/ml. The inter-day precision of the assays was also estimated and the precision was evaluated as the relative standard deviation (R.S.D.).

Extraction efficiency of HCPT from cell lysis solution was also evaluated ($n = 5$) at 2, 20 and 80 ng/ml by comparing the peak areas of an extracted sample containing a known amount of HCPT with the peak areas obtained from direct injections of the solution containing the same concentration of HCPT in pure solvent.

To investigate the stability of HCPT in blank cell lysis solution at room temperature, three concentrations (2, 20 and 80 ng/ml) of each sample were analyzed after being thawed at room temperature and kept at room temperature for 5 h.

2.7. Liposomes preparation

Liposomes were prepared by the modified thin lipid film hydration method followed by sonication [13]. Briefly, the hydrophobic excipients (Cholesterol and Soya phosphatidylcholine) and HCPT were dissolved in a mixture of methanol and chloroform (1:3, v/v) and were transferred into a round bottom flask. The organic solvent was evaporated by vacuum with rotation and a homogeneous lipid film was formed on the flask wall. The lipid film was then hydrated and sonicated. HCPT entrapment efficiency was consistently found to be greater than 80% at the drug-to-lipid ratio of 1:40 (w/w). The mean particle diameter was ∼220 nm for all liposomes preparations (polydispersity, 0.211).

2.8. Assay procedure

Eca109 cells were seeded at the density of 1×10^7/bottle to 75 cm^2 tissue culture bottles. After 12 h, the cells were

incubated with HCPT liposome and HCPT injections at 20 μg/ml for 0.5, 1, 2, 4 and 7 h, respectively. The incubation was stopped by washing the cells thrice with ice-cold PBS (pH 7.4) to stop further uptake [15]. Then the procedure was carried out according to Section 2.4. The HCPT concentration in lysis solution was determined by reversed-phase HPLC.

2.9. Statistical analysis

Statistical analyses were performed using Student's *t*-test. The differences were considered significant for p value of <0.05.

3. Results and discussion

3.1. Chromatographic specificity

At physiological pH, HCPT is unstable and hydrolysis of the lactone moiety leads to the formation of the inactive hydroxy carboxylate form [16–18]. Therefore, a bio-analytical method is required for the selective quantitation of the intact lactone form in Eca109 cells. In the present study, HPLC was employed for determination of HCPT in cytoplasm. The chromatograms of HCTP are shown in Fig. 1 under the specified chromatographic conditions. Separation of HCPT from cell lysis solution was achieved using the C_{18} column. There were no interfering peaks co-eluting with HCPT (Fig. 1A and B). Acidification of samples with H_3PO_4 and using acidic mobile phase, which can transform the carboxylate to lactone form of HCPT, were found necessary to improve the recovery. The best peak performance was achieved when the volume ratio of ACN and 0.1% triethylamine–phosphoric acid buffer (pH 3.0) was 25:75.

3.2. Validation of the method

The calibration curve for HCPT in Eca109 cells was linear over the range of 2–100 ng/ml with correlation coefficient of 0.9999. The regression equations of the calibration curves were $y = 3.43x + 2.65$. The limit of detection and the limit of quantification were 0.2 and 2 ng/ml (R.S.D. < 10%) for HCPT, respectively. According to the calibration curve, the precision and accuracy were calculated and shown in Table 1. The results showed that the intra- and inter-day coefficients of variation were less than 10% (R.S.D.). The recovery of the assay was between 86.5 and 105.2%.

Table 1
Precision and accuracy of HPLC analysis of HCPT in cell lysate samples ($n = 5$)

Added concentration (ng/ml)	Measured concentration (ng/ml)	R.S.D. (%)	Accuracy (%)
Intra-day			
2	1.73 ± 0.09	5.09	86.5
20	19.75 ± 0.51	2.58	98.8
80	80.58 ± 2.44	3.03	100.7
Inter-day			
2	1.83 ± 0.15	8.20	91.5
20	21.04 ± 1.01	4.80	105.2
80	81.48 ± 1.87	2.30	101.9

Table 2
Stability of HCPT extracted from Eca109 cell lysate samples (mean ± S.D., $n = 3$)

Target concentration (ng/ml)	HCPT (ng/ml)	
	0 h	5 h
2	1.83 ± 0.15	1.85 ± 0.06
20	19.89 ± 0.53	20.16 ± 0.37
80	81.18 ± 3.18	83.14 ± 3.25

Moreover, the extraction efficiency was determined and the results showed that the extraction efficiency for 2, 20 and 80 ng/ml HCPT from cell lysis solution was 88.9 ± 3.1, 82.1 ± 2.1 and 98.3 ± 3.0%, respectively ($n = 5$). Ethyl acetate as the extraction solvent can enhance the extraction efficiency and improve precision of the analytical method in comparison with ether. There was no significant decomposition observed in cell lysis solution spiked with various amounts of HCPT at room temperature for 5 h (Table 2). The results indicate that the method was reliable for analysis of HCPT in Eca109 cells.

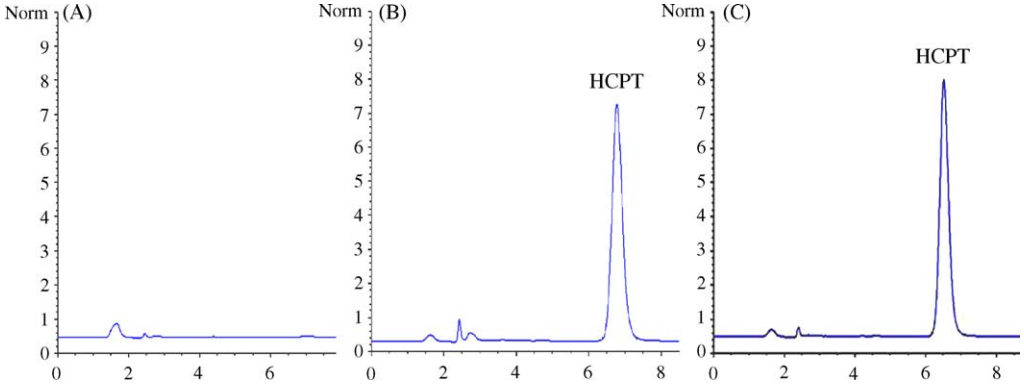

Fig. 1. Typical chromatograms of extracts of (A) blank cell lysis solution; (B) cell lysis solution spiked with 40 ng/ml HCPT and (C) HCPT accumulated in Eca109 cells at 4 h.

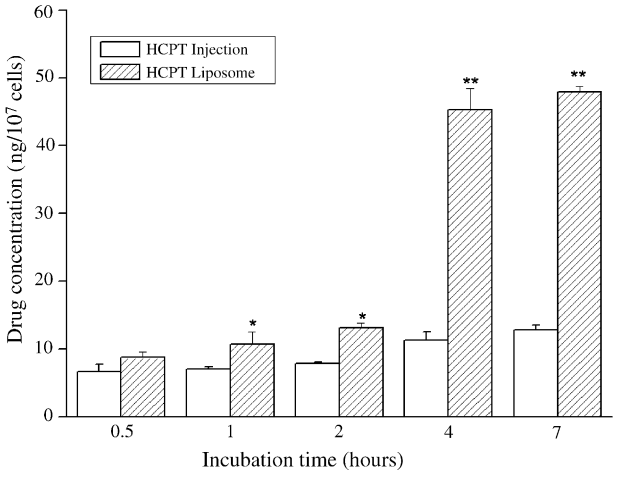

Fig. 2. Intracellular drug accumulation in Eca109 cells after incubation with 20 μg/ml HCPT injection or HCPT liposome for 0.5–7 h. Results are expressed as the amount of drug/10^7 cell. $^{*}p<0.05$, $^{**}p<0.01$ indicates significant differences between HCPT injection and HCPT liposome incubated cells.

3.3. Determination of HCPT in Eca109 cells

The amount of HCPT accumulated in Eca109 cells was determined by reversed-phase HPLC. A typical chromatogram from the cellular uptake experiment of HCPT is shown in Fig. 1C. In the present study, the cold PBS was used to wash cells in order to remove HCPT liposomes and free HCPT adhering to the cell membranes after incubation with drug containing medium. No HCPT was detected by HPLC in the final wash solution (data not shown). Thus, the values measured by the assay directly reflected the amount of HCPT that was taken up by the cells. The intracellular drug accumulation in Eca109 cells after 0.5, 1, 2, 4 and 7 h incubated with HCPT liposomes and HCPT injection is shown in Fig. 2. Eca109 cells demonstrated a time-dependent increase of drug accumulation of HCPT liposomes and HCPT injection. Liposomes associated HCPT may bypass the vesicular drug transport to improve intracellular delivery in tumor cells [14]. The results indicated that cells exposed to HCPT liposomes showed more pronounced accumulation than cells exposed to HCPT injection at 1, 2, 4 and 7 h, and the intracellular HCPT concentration was saturated within 4 h. Wang et al. [15] and Ren and Wei [19] also suggested that there is a continuous dynamic balance between cellular uptake and exocytosis when cells were incubated with drugs for a time period. The intracellular drug amount of two HCPT formulations was substantially different, suggesting that liposomes enhanced the cellular uptake of drug.

Furthermore, cytotoxic effects of both liposomal HCPT and HCPT injection were evaluated using Eca109 cells. The results indicated that cells exposed to HCPT liposomes showed more pronounced cytotoxicity than cells exposed to HCPT injection at same time period. It has been reported that the encapsulation of drug with liposomes could enhance the uptake of drug into cells and subsequently increase the cytotoxicity against tumor cells [13,20,21]. Our results also suggested that not only intracellular accumulation but also subcellular distribution might play a role in the cytotoxic potency. In order to further understand the cellular cytotoxicity of the different formulations, the study using the developed HPLC method for determining subcellular distribution of drug is under way.

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