Determination of Hydroxycamptothecin by Flow Injection Analysis With Chemiluminescence Detection

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An novel analytical method consisting of flow-injection sampling and chemiluminescence (CL) detection for determination of anticancer drug hydroxycamptothecin (HCPT) is described. It is based on the CL reaction of potassium permanganate–formaldehyde–HCPT system. The formaldehyde-sensitized CL emission mechanism was proposed for HCPT–potassium permanganate (KMnO₄)–HCOH systems by comparing the fluorescence emission with CL spectra. Illuminant was the single-state bimolecular oxygen, ¹O₂(¹Δ_g), which was from ¹O₂(¹Δ_g) produced in the reaction system, and emits CL spectra at 639 nm. The presence of formaldehyde can sensitized CL emission, because it may be to

accelerate the generation of ¹O₂(¹Δ_g). The optimum conditions for CL emission were investigated and optimized. The CL intensity was correlated linearly with concentration of HCPT in the range of 0.010–60.0 mg L⁻¹. The determination limit is 0.006 mg L⁻¹. The relative standard deviation is 1.6% for 11 parallel measurements of 0.20 mg L⁻¹ HCPT standard solution. The proposed method has been applied for the determination of HCPT in pharmaceutical preparations, serum, and urine. The results of the quantitative analysis for HCPT studied using the developed method showed a good agreement with provided by using an official method. J. Clin. Lab. Anal. 21:356–362, 2007. © 2007 Wiley-Liss, Inc.

Key words: hydroxycamptothecin; chemiluminescence; flow-injection; pharmaceutical analysis; biological fluids

INTRODUCTION

Hydroxycamptothecin (HCPT) is a water-soluble derivative of camptothecin (CPT) (1) and exhibits a remarkable antihepatoma effect. CPT is a potent antitumor alkaloid extracted from the Chinese tree *Camptotheca acuminata* (2). The antitumor mechanism of the compound is based on the irreversible inhibition of deoxyribonucleic acid (DNA) topoisomerase I (TOPO I) (3–5). However, clinical use of CPT was discontinued due to its high toxicity, and water-soluble HCPT was developed. HCPT has been used to cure liver carcinoma, urinary bladder carcinoma, and colorectal cancer, etc. in China (6). HCPT can exist in two forms (Fig. 1): carboxylate and lactone. At pH less than 4, α-hydroxy-γ-lactone structure predominates. At more alkaline pH, lactone form can transfer to carboxylate form by the reversible hydrolysis. Recently, clinical research has shown that some metabolites and derivatives of the

anticancer drugs have stronger bioactivity than the anticancer drugs. Quality control of drug dosage, its monitoring in biological fluids, and research of anticancer drug's metabolism and action are important analytical tasks. Therefore it is necessary to establish sensitive analytical technique.

Over the past years, several method for the quantification of HCPT have been reported, including high-performance liquid chromatography (HPLC) methods

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Fig. 1. Chemical structure of two forms of HCPT.

(7–9), capillary electrophoresis methods (10,11), electrochemical method (12), and ultraviolet (UV) method (13). The process methods were complicated, the sensitivity was low or the analytical conditions were harsh, or the instrument was expensive.

Chemiluminescence (CL) is becoming a powerful analytical tool with widespread application in various fields owing to its high sensitivity, wide dynamic range and simple instrumentation (14,15). A critical review was presented for the use of acidic potassium permanganate to generate CL during the oxidation of both organic compounds and inorganic species (16). Several studies have reported that the very sensitive CL emission was generated by oxidation of hydroxyl-containing drugs with potassium permanganate (KMnO_4) in acidic medium (17,18). Formaldehyde could enhance the CL emission intensity of such system, which laid a foundation of using HCOH as sensitized agent in CL analysis, and stimulated researchers to explore their application as sensitized agent in analysis of melatonin and some of its derivatives (19), hydralazine (20), naftopidil (21) and estrogens (22). Although CL method has been used for pharmaceutical analysis, but there was few report on CL method for detecting HCPT.

The main purpose of this paper is to develop a new sensitized CL system based on the use of acidified KMnO_4 as oxidant in the presence of formaldehyde for determination of the anticancer drug HCPT. The proposed sensitized CL system has been successfully applied for the determination of HCPT in the injection and biological fluid with satisfactory result. The CL mechanism was developed.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical-reagent grade. Water was purified with an XGJ-30 water purified system purchased from the Yongsheng Company in Beijing, China. HCPT (Institute of Medicinal Biotechnology, Beijing, China) stock standard solution (500 mg L^{-1}) was prepared by dissolving 25.0 mg HCPT in 2 mL 0.1 mol L^{-1} NaOH and diluting with deionized water to 50 mL. More dilute HCPT solutions were freshly prepared by diluting the stock solution with deionized water. HCPT for injection lyophilized powder were provided by Shenzhen Main Luck Pharmaceuticals, Inc. (Guangdong, China). A stock solution of KMnO_4 ($5 \times 10^{-2} \text{ mol L}^{-1}$) was prepared by dissolving a weighed amount of KMnO_4 in water and diluting to volume. A stock solution of HCOH (2.5%) and solution of H_2SO_4 (3 mol L^{-1}) were prepared daily.

Apparatus

The MPI-B-FI-CL analysis system (Xi'an Ruimai Electronic Equipment Company, China), which is automatically operated by a computer, consists of two peristaltic pumps working at a constant flow rate ($30 \text{ revolutions per minute [rev. min}^{-1}]$) and a six-way injection valve with a sample loop ($120 \mu\text{L}$). Polytetrafluoroethylene (PTFE) tubing (0.8 mm inner diameter [i.d.]) was used to connect all components in the flow system. Because there is no reaction between KMnO_4 solution and H_2SO_4 solution, the two solutions were merged previously and used as the carrying fluid. The mixture flowed into a flow cell through a three-way pipe. A mixture of sample and HCOH solution was injected from a sample valve, the CL signal is produced immediately. The flow cell is a twisty glass tube, which is used to produce a large surface area exposed to the adjacent photomultiplier tube (PMT; Amamatsu, Japan). The signal from the CL reaction was recorded by an analyzer. Spectrophotometric method was used with a UV-265 spectrophotometer (Shimadzu, Japan). Fluorescence spectra were recorded with RF-5301PC spectrofluorophotometer (Shimadzu, Japan). The flow-injection CL analysis system for the determination of HCPT is shown in Fig. 2.

Sample Preparation

Injection Sample Treatment

Sample solutions for analysis were prepared as follows. The average bottle weights were calculated from the weight of 10 bottles that were randomly selected from the same group. An accurately weighed

Fig. 2. Schematic diagram of flow-injection CL analysis system. P, peristaltic pump; V, sampling inlet valve; C, flowing cell; PMT, photomultiplier tube; AMP, amplifier; HV, high voltage; R, recorder; W, waste; a, HCPT solution; b, HCOH solution; c, KMnO_4 solution; d, H_2SO_4 solution.

portion of each homogenized sample containing 5 mg of HCPT was dissolved with 2 mL of 0.1 mol L^{-1} NaOH and water in a small beaker. The solution was filtered and the residue was washed with water several times, then transferred into a 25-mL calibrated flask and diluted to the volume with water. Working solutions were prepared by appropriate dilutions so that the final concentration was in the linear range.

Serum Treatment

A 1-mL volume of serum sample was deproteinized by adding 1.0 mL 10% trichloroacetic acid (CCl_3COOH) in a centrifuge tube, which was then centrifuged for 15 min at $4,000 \text{ rev. min}^{-1}$. The supernatant liquor was tested the concentration of HCPT. Patient samples of serum were kindly provided by clinical laboratory, Taian Tumor Hospital.

Procedure

As shown in Fig. 2, all solutions were continuously pumped into the manifold. HCPT solution was first mixed with HCOH solution via a Y-piece prior to the six-way valve, and then $120 \mu\text{L}$ of the mixture was injected into a mixed stream of KMnO_4 and H_2SO_4 solutions to produce CL. The signal from the CL reaction was recorded by an analyzer. Calibration graphs were constructed by plotting the intensity (peak height) of the CL signal versus the concentration of HCPT.

RESULTS AND DISCUSSION

Optimization of Experimental Condition

A series of experiments were conducted to establish the optimal conditions for the production of maximum

Fig. 3. Effect of sulfuric acid concentration on relative CL intensity. ($\text{KMnO}_4 = 5 \times 10^{-4} \text{ mol L}^{-1}$, $\text{HCOH} = 2\%$, $\text{C}(\text{HCPT}) = 10 \text{ mg L}^{-1}$).

CL emission for the KMnO_4 -HCPT-HCOH system. The chemical variables and flow-injection variables were investigated, respectively.

Effect of Sample Volume and Flow Rate

The role of sample volume and flow rate is critical, sample volume was too small or too large, maximum CL could not be obtained. The highest emission occurred if the injected sample volume was $120 \mu\text{L}$. The CL intensity increased with increasing flow rate. However, a flow rate of 3.0 mL min^{-1} for all solutions is recommended because of greater precision and economy in the use of reagents.

Choice of Inorganic Acids

The nature and concentration of the acid used in the reaction has a very significant influence on the CL emission intensity. Therefore, several acids, such as HCl, H_2SO_4 , HNO_3 , H_3PO_4 , and $\text{H}_6\text{P}_4\text{O}_{13}$, were added in the KMnO_4 solution to test the effect of acid on the CL signal, respectively. The highest emission was observed from H_2SO_4 -treated KMnO_4 solutions, and the signal was stable. Hence, H_2SO_4 was chosen for further work. The concentration of H_2SO_4 in KMnO_4 solution was subsequently optimized (Fig. 3); 3.0 mol L^{-1} H_2SO_4 was optimal and was used for further experiments.

Effect of KMnO_4 Concentration

In this CL system, KMnO_4 was used as the oxidant. The KMnO_4 concentration not only had an effect on the

Fig. 4. Effect of KMnO_4 concentration on relative CL intensity. ($\text{H}_2\text{SO}_4 = 3.0 \text{ mol L}^{-1}$, $\text{HCOH} = 2\%$, $\text{C}(\text{HCPT}) = 10 \text{ mg L}^{-1}$).

Fig. 5. Effect of formaldehyde concentration on relative CL intensity. ($\text{KMnO}_4 = 2.5 \times 10^{-4} \text{ mol L}^{-1}$, $\text{H}_2\text{SO}_4 = 3.0 \text{ mol L}^{-1}$, $\text{C}(\text{HCPT}) = 10 \text{ mg L}^{-1}$).

sensitivity, but also influenced the linear range for the assay. The concentration of KMnO_4 was expected to affect the CL intensity. Therefore, the dependence of the CL intensity on the KMnO_4 concentration was investigated for 10.0 mg L^{-1} HCPT. The results (Fig. 4) showed that the maximum CL intensity was obtained when the KMnO_4 concentration was $2.5 \times 10^{-4} \text{ mol L}^{-1}$. Then, the KMnO_4 concentration of $2.5 \times 10^{-4} \text{ mol L}^{-1}$ was chosen as optimum.

Effect of Sensitizers

The KMnO_4 -HCPT system could only produce a weak CL emission. Various compounds, such as rhodamine B, HCOH , H_2O_2 , Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$, and $\text{Na}_2\text{S}_2\text{O}_4$, were tested as sensitizers for the CL system of KMnO_4 -HCPT. It was found that only HCOH enhanced the CL signal for the KMnO_4 -HCPT system. The effect of HCOH concentration on the CL intensity was studied in the range of 0.5–3%, as shown in Fig. 5. The CL intensity was enhanced with increasing HCOH concentrations. The maximum CL intensity was obtained when the HCOH concentration was 2.5%. Hence, the HCOH working concentration was fixed at 2.5%.

Interference Studies

The influence of some common excipients used in drugs, metal ions in human body and several organic compounds on the CL intensity was investigated for the determination of 4.0 mg L^{-1} HCPT by comparing with the CL emissions obtained using HCPT solution alone or HCPT with foreign species added. Tolerance was

defined as the amount of foreign species that produced an error not exceeding $\pm 5\%$ in the determination of HCPT. The results are listed in Table 1. There were interference from equal amounts of hemoglobin, myoglobin, vitamin B_1 , I^- , and Fe^{2+} .

Kinetic Characteristics of the CL Reaction

The CL kinetic characteristics of the reactions of HCPT, HCOH , and acidic KMnO_4 in solution were studied in detail. It was determined that the reaction rate in solution was very fast; from reagent mixing to peak maximum only 3 sec was needed, and it took 9 sec for the signal to return to zero again.

Performance of the System for HCPT Measurements

Under optimum conditions as those described above, the linearity of the calibration graph of HCPT was examined in the range from 0.010 to 60.0 mg L^{-1} . The calibration graph was consists of four parts in order to improve the veracity. The basic parameter of the calibration graph is listed on Table 2. The detection limit (3σ) for HCPT is 0.006 mg L^{-1} . The relative standard deviation was 1.6% for 11 determinations of 0.20 mg L^{-1} HCPT. The RSDs of the intraday variations were 1.8% and 1.5%, and the interday RSDs were 2.0% and 1.6% in the concentrations of 0.10 mg L^{-1} and 6.0 mg L^{-1} HCPT for seven determinations, respectively. It is indicated that the proposed CL systems have good linearity, higher sensitivity, and precision.

TABLE 1. Maximum tolerable concentration ratios with respect to 4 mg L⁻¹ HCPT for quantitative determination

Substance species	Tolerable concentration ratio
Magnesium stearate, lactose, galactose, sodium citrate, starch, fructose, sucrose, glucose, dextrin, sodium benzoate	100
Polyglycol	25
Zn ²⁺ , Ba ²⁺ , Al ³⁺ , Mg ²⁺ , Ni ²⁺ , Ca ²⁺ , Cu ²⁺ , Co ²⁺	100
Fe ³⁺ , Br ⁻	10

TABLE 2. Linear range and regressive equations of the calibration curves

HCPT concentration (mg L ⁻¹)	Regressive equation I = aC + b	Correlation coefficient r
0.010–0.10	I = 1.4C + 41.8	0.9959
0.10–1.0	I = 9.1C + 39.4	0.9988
1.0–10.0	I = 70.9C + 74.2	0.9986
10.0–60.0	I = 401.0C + 400.9	0.9992

Sample Analysis

Pharmaceutical Analysis

The proposed method was applied to the determination of HCPT in injections. The obtained results are summarized in Table 3 along with labeled contents. The results agree well with those obtained by the UV-method (13). There was no significant difference between the labeled contents and the results obtained by the proposed method.

Recovery Experiments for Drug

In order to evaluate the validity of the proposed method for the determination of HCPT in pharmaceutical, recovery studies were carried out on samples to which known amounts of HCPT were added. The results are given in Table 4. The recoveries were in the range of 95.0–103.3%.

Recovery Experiments for Serum and Urine Sample

It was reported (23) that the concentration of HCPT in the serum was 32.5–50.2 ng mL⁻¹ after injection of 30 mg HCPT. To make the sample concentration of the drug within the linear range of determination, the serum sample was diluted appropriately. The standard addition method was used to avoid matrix effects. Different cancer patients were injected with 30 mg HCPT, and their serum samples were tested within 2 hr. The results

TABLE 3. Determination results of HCPT in sample

Batch number	Nominal (mg/bottle)	Proposed method (mg/bottle)	RSD (%; n = 7)	UV method (mg/bottle)
0410H1	5	5.04	1.8	5.10
0502H2	5	4.98	1.7	5.03

TABLE 4. Recovery experiments

Content (mg L ⁻¹)	Added (mg L ⁻¹)	Found (mg L ⁻¹)	Recovery (%; n = 7)
0.27	0.2	0.46	95.0
	0.4	0.68	102.5
	0.6	0.85	96.6
2.53	2.0	4.55	101.0
	4.0	6.49	99.0
	6.0	8.73	103.3

TABLE 5. Determination result in different patient serum samples

Sample #	Content (0.010 mg L ⁻¹)	Added (0.010 mg L ⁻¹)	Found (0.010 mg L ⁻¹)	Recovery (%)	RSD n = 5 (%)
1	6.8	5.0	12.1	106.0	1.8
2	7.2	5.0	12.3	102.0	1.9
3	8.1	5.0	12.9	96.0	1.7
4	4.9	5.0	10.1	104.0	2.0
5	5.8	5.0	10.7	98.0	1.8

for the content and the recoveries are summarized in Table 5.

Possible CL Mechanism of the Reaction

The fluorescence and CL spectra were examined in order to obtain more information about the enhanced CL mechanism. The fluorescence emission spectra of HCPT and the KMnO₄-HCPT system were observed. As shown in Fig. 6, they show different emission spectra. The native fluorescence emission of HCPT shows two broad peak centers at 440 and 550 nm. When KMnO₄ was added into the HCPT solution, the maximum wavelength was always at 440 nm (it may be the oxide of HCPT) and 550 nm, but the peak intensity at 440 nm increased and at 550 nm it greatly decreased. After HCOH was added into the potassium permanganate (KMnO₄) and HCPT solution, the fluorescence emission showed one broad peak with the center at

Fig. 6. Fluorescence emission spectra of HCPT (B), HCPT-KMnO₄ (C), HCPT-KMnO₄-HCOH (D), HCPT-HCOH (E). ($\lambda_{\text{ex}} = 363 \text{ nm}$, $\text{KMnO}_4 = 2.5 \times 10^{-4} \text{ mol L}^{-1}$, $\text{HCOH} = 2.5\%$, $\text{C}(\text{HCPT}) = 0.5 \text{ mg L}^{-1}$).

440 nm; the peak intensity is higher than in the KMnO₄-HCPT system, so it is possibly enhanced by HCOH. The mixing with HCOH and HCPT, they are similar to the fluorescence emission spectrum of KMnO₄-HCOH-HCPT, the fluorescence emission spectra center always at 440 nm.

To get an idea about the reaction product generating the CL, the CL spectrum of the reaction was examined by a series of interference filters. The CL spectra in the KMnO₄-HCOH-H₂SO₄ solution in the absence and presence of HCPT were also recorded in the range 360–730 nm. When HCPT was absent, no obvious CL spectrum was observed. The CL spectrum in the presence of HCPT showed a peak at 639 nm (Fig. 7). The peak coincided with the $\text{ClO}^{-1}\text{-H}_2\text{O}_2$ system of $^1\text{O}_2\text{O}_2(^1\Delta_g\Delta_g)$ (643 nm) (24), and good agreement with KMnO₄-HCOH-methotrexatum (25). Therefore, the possible CL reaction scheme of the reaction may be attributed to the following reactions in its simplest form:

CONCLUSION

The proposed flow-injection CL method has a simple, rapid, inexpensive and high sensitivity for the determi-

Fig. 7. CL spectra of HCPT-KMnO₄-HCOH-H₂SO₄. ($\text{KMnO}_4 = 2.5 \times 10^{-4} \text{ mol L}^{-1}$, $\text{H}_2\text{SO}_4 = 3 \text{ mol L}^{-1}$, $\text{HCOH} = 2.5\%$, $\text{C}(\text{HCPT}) = 200 \text{ mg L}^{-1}$).

nation of HCPT. The weak luminescence analyzer combined with the flow-injection system can rapidly attain high measurement; up to a sample throughput of 60 samples per hr was attained. Furthermore, the apparatus are cheaper than those of spectrophotometry, capillary electrophoresis, and HPLC. The CL reaction is of adequate sensitivity for the determination of low analyte contents in the samples with wider linearly range and lower detection limit. Therefore, the proposed method has a simple and suitable for routine determination of the drugs

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