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Short communication

Liquid chromatography determination of 10-hydroxycamptothecin in human serum by a column-switching system containing a pre-column with restricted access media and its application to a clinical pharmacokinetic study

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Abstract

A simple, rapid, sensitive column-switching HPLC method is described for the analysis of the 10-hydroxycamptothecin (HCPT) in human serum. A pre-column containing restricted access media (RAM) is used for the sample clean-up and trace enrichment and is combined with a C_{18} column for the final separation. The analytical time is 8 min. The HCPT is monitored with fluorescence detector, excitation and emission wavelengths being 385 and 539 nm, respectively. There is a linear response range of 1–1000 ng/ml with correlation coefficient of 0.998 while the limit of quantification is 0.1 ng/ml. The intra-day and inter-day variations are less than 5%. This analytic procedure has been applied to a pharmacokinetic study of HCPT in clinical patients and the pharmacokinetic parameters of one-compartment model are calculated.

Keywords: Pharmacokinetics; Restricted access media; 10-Hydroxycamptothecin

1. Introduction

Camptothecin (CPT) is a natural alkaloid extracted from Camptotheca Acuminata Decne in 1960s [1]. Displaying promising activity of anti-tumor, the drug and its semi-synthetic analogues have been paid more attention to. In 1990s, several CPT analogues were synthesized, including irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) (CPT-11) 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]. All CPT analogues can undergo a reversible, pH-dependent hydrolysis. 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. The factors influencing the lactone–carboxylate equilibria of

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camptothecins, including proteins, enzymes, fluids and otherwise, are more complicated both in vitro and in vivo.

Several HPLC methods were reported to analyze CPT drugs in human plasma [4–11]. The sample pre-treatment was required to remove proteins before injection. The means included precipitation by organic solvent, liquid–liquid extraction, solid-phase extraction and so on, resulting in the decrease of efficiency and time wasting. It was not reported to determine the concentration of CPT analogues by column-switching HPLC system.

Different kinds of restricted access media (RAM) were developed for the direct injection analysis of biological fluids [12,13]. Their features can be described as follows: (1) There is a hydrophilic external surface to avoid denaturing proteins. (2) The pore of packing is small enough for proteins to be eluted without any retention by size exclusion mechanism. (3) The internal surface of packing is of hydrophobicity to a certain extent by means of which small molecules are retained and separated. We have built a method for simultaneous determination of the carboxylate and lactone forms of 10-hydroxycaptothecin in human serum by RAM–HPLC [14].

The HPLC column-switching technique is widely used for the direct injection analysis of biological fluids. In the column-switching system, the sample clean-up and trace enrichment are completed in on-line, improving sensitivity and not needing pre-treatment [15]. The RAM is used not only as packing for analytical columns, but also as packing for pre-column of column-switch. In this case the pre-column can be used repeatedly many times for removing the proteins of clogging column [16,17].

The purpose of this work is to develop a HPLC method for the analysis of the HCPT in human serum by column-switching system containing pre-column with RAM and by combining analytical column with C_{18}, which can be used in HCPT pharmacokinetic study. HCPT, its analysis of bio-fluids and pharmacokinetic parameters being hardly reported [18,19], can exist in two forms: carboxylate and lactone. The monitoring of total HCPT has essentially the same clinical significance as the monitoring of lactone or carboxylate HCPT, because the pharmacokinetics of total HCPT is correlated significantly with that of lactone or carboxylate HCPT, respectively [20,21]. Thus we should determine total HCPT and study its clinical pharmacokinetics in patients.

2. Experimental

2.1. Materials

HCPT and CPT were provided by Feiyun Pharmaceutical Company (Huangshi, Hubei, China). HPLC grade acetonitrile (ACN) was obtained from Siyou (Tianjin, China). All other chemicals were of analytical grade from a variety of suppliers. The aqueous solutions were prepared by using double distilled water.

2.1.1. HCPT stock solution

HCPT was dissolved in an appropriate amount of dimethylsulfoxide (DMSO). The solution was stored at $-20\,^{\circ}C$. HCPT standard solution: The stock solution was diluted with DMSO–0.01 M phosphate buffer, pH 2.5 (50:50, v/v) for lactone form. The solution was equilibrated at ambient for 30 min and was used in the same day when they were prepared.

2.2. Chromatography

2.2.1. Clean-up and trace enrichment

A HPLC system consisted of LC-6A pump (Shimadzu, Kyoto, Japan), Rheodyne 7125 injection valve with 100 μl loop (Catati, CA, USA), SPD-6A UV spectrophotometric detector (Shimadzu, Kyoto, Japan). Analysis: A HPLC system consisted of Waters 600E pump (Waters, Milford, MA, USA), Rheodyne 7725 switching valve (Catati, CA, USA), CTO-6A column oven and RF-530 fluorescence detector (Shimadzu, Kyoto, Japan), The output was connected to Autoscience chromatographic workstation (Autoscience, Tianjing, China).

The chromatographic condition was set up as follows: pre-column, 30 mm × 4.6 mm, RAM–MFP packing, 5 μm, method of preparation and packed in [22]; mobile phase, ACN:25 mM phosphate buffer (pH 3) = 5:95 (v/v), flow-rate, 0.8 ml/min; UV detector at 280 nm; injection volume, 100 μl; time of clean-up and trace enrichment, 5 min. Analytical column, 3.9 mm × 150 mm, Symmetry C_{18}, 5 μm (Waters, Milford, MA, USA); mobile phase, ACN:25 mM

phosphate buffer (pH 3) = 60:40 (v/v), flow-rate, 0.8 ml/min; degas with helium on line, 30 ml/min; fluorescence detection, excitation 385 nm, emission 539 nm; temperature, 30 °C, back-flush time, 3 min, analytical time, 5 min.

2.3. Standard serum samples

Blank human serums without drugs were provided by healthy volunteers, which were diluted by adding same volume 0.1 M phosphate buffer (pH 1.8).

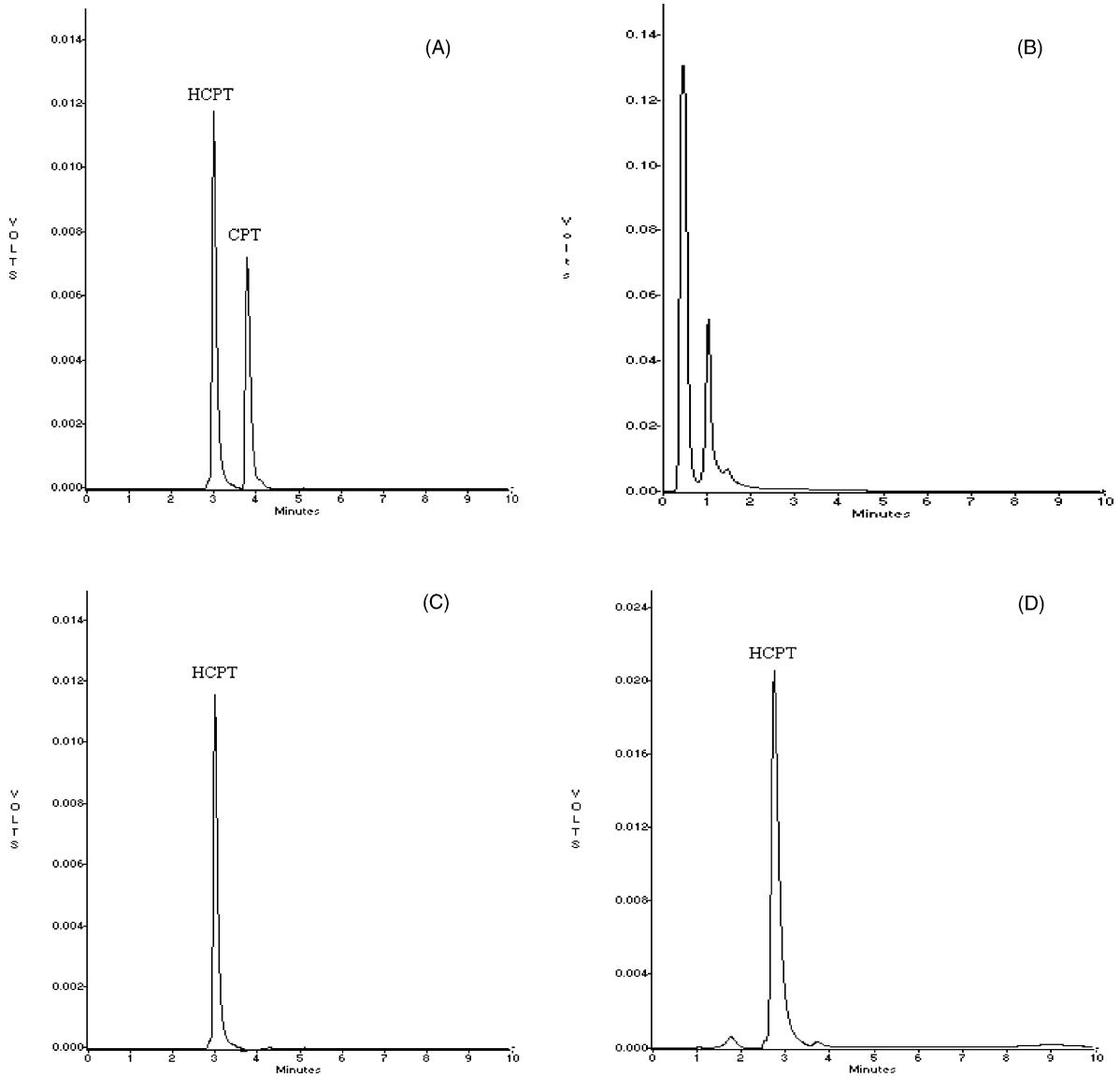

Fig. 1. Chromatograms: (A) standard solution, mixture of HCPT (0.1 μg/ml) and CPT (0.3 μg/ml), fluorescence detector after analytical column; (B) and (C) standard serum sample, HCPT (0.1 μg/ml), UV detector after pre-column in (B), fluorescence detector after analytical column in (C); (D) the sample obtained from a patient after intravenous administration of HCPT at a dose of 10 mg, fluorescence detector after analytical column. Chromatographic conditions: see Section 2.2.

Standard serum samples of HCPT: 30 μl of each in HCPT standard solution series was added into 270 μl of diluted blank serum. The final concentration of standard serum samples was 0.1, 1, 2, 10, 50, 100, 250, 500, 1000 ng/ml.

2.4. Calibration curve

The standard serum samples were directly injected into the HPLC system. The peak areas were plotted against the concentrations to obtain the calibration curve for HCPT.

2.5. Clinical pharmacokinetic studies

After operation, seven chemical treating patients with stomach or intestinal cancer came from Department of Neoplasm, General Hospital of Lanzhou Command of PLA, whose sex was not distinguished, ages were 20–60 years and weight was 67 ± 10 kg, having normal hepatic and renal function. After every patient was administrated intravenously with dosage of 10 mg HCPT in 10 min, blood samples were collected at 0, 10, 30, 60, 120, 240, 720 and 1440 min and then centrifuged, respectively. Eighty microliters of the

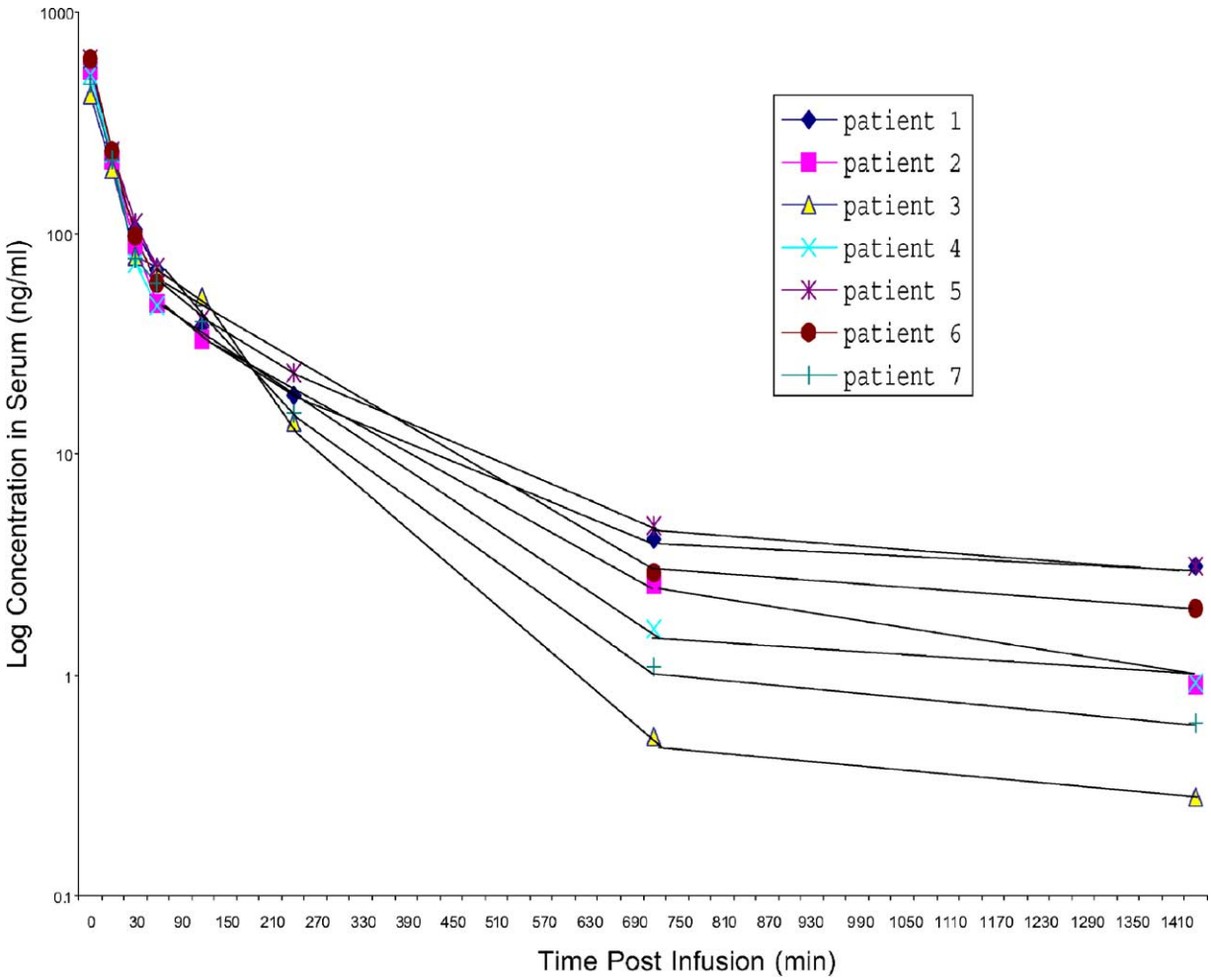

Fig. 2. Concentration–time course of HCPT determined in serum of patients (dose 10 mg, infusion time 10 min).

centrifuged serum was added to 80 μl of 0.1 M phosphate buffer (pH 1.8), the tube being vortex-mixed for 30 s and stored at −20 °C until analysis.

The pharmacokinetic parameters of HCPT were calculated using 3P97 programs recommended by the Chinese Pharmacological Society.

3. Results and discussion

3.1. Column-switching

Twenty-five millimolars phosphate buffer (pH 3)/ACN was selected as mobile phase to eluate only the pre-column. After 50 μl of blank serum was directly injected into pre-column using the first time 5 times continuously, the changes of peak area for proteins by measuring UV absorbance at 280 nm were observed: 918276 → 1539684 → 1991698 → 2182153 → 2189751. With the numbers of injection increasing, the peak areas have a tendency from increasing to stable level. Some uncovering hydrophobic groups on the surface of the RAM packing were blocked by proteins after many injections.

Different ACN concentrations in the mobile phase have a great influence to HCPT retention in chromatographic separation of the pre-column. The weak mobile phase (ACN:25 mM phosphate buffer (pH 3) = 5:95, v/v), for loading serum sample onto the RAM pre-column, can exclude most of the proteins and hydrophilic endogenous compounds effectively to waste and simultaneously retain the analytes completely in 5 min. When the valve was switched, the retained analytes from the RAM pre-column were swept by the strong mobile phase (ACN:25 mM phosphate buffer (pH 3) = 60:40, v/v) to the top of the analytical column in a back-flush in 3 min. The switching valve was changed to recondition the RAM pre-column with the weak mobile phase to be ready for the next injection. In the meantime, the separation was performed by the strong mobile phase and analytical column in 5 min.

3.2. Chromatogram

The chromatograms are shown in Fig. 1 under the specified chromatographic conditions. Fig. 1A is a chromatogram of mixture of HCPT and CPT standard solution by fluorescence detector. Fig. 1B and C are chromatograms of the standard serum samples, the Fig. 1B representing UV-absorption of proteins in the serum by the pre-column, and the Fig. 1C representing fluorescence determination of HCPT by the analytical column. Fig. 1D is a chromatogram of the blood sample taken from a patient after intravenous administration of HCPT at a dose of 10 mg. It is clearly explained from the chromatograms that hydrophilic endogenous compounds such as proteins were eluted by the pre-column and HCPT in analytes was separated rapidly by the analytical column with retention time 3 min only.

In the determination of HCPT and its derivatives, acidification and dilution of samples with 0.1 M H_3PO_4 (pH 1.8) and using acidic the mobile phase (pH 3), in order to transform the carboxylate to lactone form of HCPT, can determine total HCPT concentration and elute the serum proteins smoothly. It is testified by our experiment that the pre-column was used for many repetitive injections of serum samples (about total 10 ml, non-diluted) with increase in back-pressure about 20 kg/cm^2. The pre-column shall be replaced after over total 15 ml of serum samples were injected repeatedly.

3.3. Calibration curve

Under the specified chromatographic conditions, the peak areas were plotted against the standard serum concentrations of HCPT to give the calibration curve: $Y = 0.001756X + 0.400$, $R = 0.998$, X and Y were

Table 1
Precision and accuracy of HPLC analysis of HCPT in serum ($n = 3$)

Added concentration (ng/ml)	Measured concentration (ng/ml)	R.S.D. (%)	Accuracy (%)
Intra-day			
2	1.8	8.2	90.0
10	9.7	3.1	97.0
100	102.3	0.2	102.3
500	507.6	0.1	101.5
Inter-day			
2	2.2	8.7	110.0
10	9.7	4.2	97.0
100	103.1	0.2	103.1
500	510.3	0.2	102.1

Table 2
Pharmacokinetic parameters of HCPT after intravenous infusion in seven patients (dose 10 mg)

Parameter	$\bar{x} \pm$ S.D.
C_0 (ng/ml)	532 ± 46
K_e (1/min)	0.0760 ± 0.0107
V_c (ml/kg)	290 ± 51
$T_{1/2}$ (min)	9.28 ± 1.34
AUC ((ng/ml)·min)	7005 ± 435
CL ((ml/kg)/min)	22.0 ± 3.0

C_0: serum–drug concentration at time zero after intravenous bolus dose; K_e: elimination rate constant; V_c: apparent volume of distribution; $T_{1/2}$: half-life of drug; AUC: area under the serum concentration–time course; CL: clearance of drug.

the peak area and the concentration, respectively, the concentration ranges being 1–1000 ng/ml. The limit of quantification (LOD) for HCPT is 0.1 ng/ml (S/N $\geq$ 3) and this method has a high sensitivity. When a 500 μl of loop is installed and a large volume of sample is injected according to the need of analysis, the determining sensitivity will be higher (LOD 20 pg/ml).

3.4. Validation

According to the calibration curve, the precision and accuracy were calculated and shown in Table 1. The intra-day precision and accuracy are 2.9 and 97.7% and the inter-day precision and accuracy are 3.3 and 103.1%, respectively. The recovery of HCPT from serum is 99.0% that is determined by comparing the areas of standard solution with those of standard serum sample containing the same amount of HCPT. For eliminating the need for biological sample preparation, the method has almost 100% recovery and good reproducibility.

Compared with the previous RAM–HPLC system [14], the present method has improved sensitivity and given much flexibility in the choice of functionality of the stationary phase and mobile phase in analytical column, but requiring additional equipment.

3.5. Pharmacokinetic parameters

The concentration–time course of HCPT is illustrated in Fig. 2 and the pharmacokinetic parameters are shown in Table 2. Pharmacokinetic analysis revealed that, following intravenous administration, the serum disappearance curve for HCPT could be best described by a one-compartment model, HCPT was distributed widely and eliminated at a fairly rapid rate in the human.

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