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Measurement and pharmacokinetics of unbound 20(*S*)-camptothecin in rat blood and brain by microdialysis coupled to microbore liquid chromatography with fluorescence detection

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Abstract

To characterize the pharmacokinetics of protein-free camptothecin in blood and brain we implanted microdialysis probes into the jugular vein and striatum of rats for unbound drug sampling and determination. Camptothecin (2 or 5 mg/kg, i.v., $n=6$) was then administered from the femoral vein, and microdialysates were collected from blood and brain of both sites and assayed by a validated microbore scale high-performance liquid chromatographic method. The mobile phase consisted of methanol–100 m*M* monosodium phosphoric acid (35:65, v/v, pH 2.5) with a flow-rate 0.05 ml/min. The fluorescence response for camptothecin was observed at excitation and emission wavelengths of 360 and 440 nm, respectively. Pharmacokinetic parameters were calculated from the corrected data for dialysate concentrations of camptothecin versus time. The results suggest that the pharmacokinetics of unbound camptothecin in blood and brain can be fitted best to a two- and one-compartment model, respectively. Camptothecin rapidly entered the extracellular fluid of brain striatum at 10 min following camptothecin administration.

Keywords: Camptothecin

1. Introduction

Camptothecin was originally isolated from the wood and bark of *Camptotheca acuminata*, and it has been shown to have significant cytotoxic and anti-tumor effects [1]. The mechanism of camptothecin may be to inhibit topoisomerase I, an enzyme that is required for the replication and transcription of cells [2,3]. Despite numerous studies describing the plasma pharmacokinetics of camptothecin or its derivatives [4–7], the pharmacokinetics of unbound camptothecin in the blood and brain have not previously been described. Microdialysis is a technique that enables to measure the protein-unbound drug concentrations as a function of time and is therefore suitable for the pharmacokinetic study. The total drug concentration in the biological fluid does not immediately reflect concentrations at the cellular level for the pharmacological response. Therefore, monitoring the drug concentration in the interstitial space is crucial to understanding the time course of biological activity of the drug.

Of the several techniques that have been employed to study drug transport to the brain [8], one of the more traditional methods is the brain tissue homogenate method. Microdialysis, on the other hand, is an

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in vivo sampling technique that offers several advantages, chief of which for the present purposes is the opportunity to continuously and simultaneously monitor drug concentrations in the same animals when coupled to an adequate analytic technique [9,10]. Microdialysis procedures measure only protein-free drugs, because protein molecules are too large to pass through the dialysis membrane (molecular mass cut-off 13 000). These procedures therefore allow determination of drug concentrations from protein unbound and extracellular space in most tissues [11–13]. However, depending on the sensitivity of the analytical technique, finite volumes of dialysates are required and the measured drug concentrations therefore actually represent the total drug concentrations for a given time period.

For this study, we constructed blood and brain microdialysis probes, then inserted them into the rat jugular veins and brain striata for sampling of camptothecin from biological fluids after camptothecin was given intravenously. The quantitative analysis was carried out using validated microbore high-performance liquid chromatography (HPLC) methods with fluorescence detection. The limit of quantification was 1 ng/ml. Microdialysis therefore appears to be a suitable technique for delivering a drug within a specific site for additional pharmacokinetic studies.

2. Experimental

2.1. Reagents

20(*S*)-Camptothecin (Fig. 1) was purchased from Aldrich (Milwaukee, WI, USA). The chromatographic solvents and chemical reagents were obtained from BDH (Poole, UK) and Sigma (St. Louis, MO, USA), respectively. Triple deionized water from Millipore (Bedford, MA, USA) was used for all preparations.

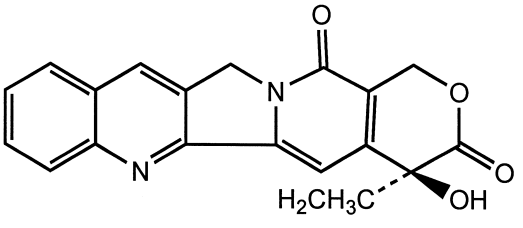

Fig. 1. Chemical structure of 20(*S*)-camptothecin.

2.2. Liquid chromatography

The liquid chromatographic system consisted of a chromatographic pump (Bioanalytical Systems, BAS PM-80, West Lafayette, IN, USA), an on-line injector (CMA 160, Stockholm, Sweden) equipped with a 10-μl sample loop and a fluorescence detector (Linear Model LC305, San Jose, CA, USA). Dialysates were separated using a reversed-phase C_{18} microbore column (150×1 mm I.D.; particle size 5 μm, Bioanalytical Systems) maintained at an ambient temperature to perform the ideal chromatographic phase. The mobile phase consisted of methanol–100 m*M* monosodium phosphoric acid (35:65, v/v, pH 2.5) with a flow-rate 0.05 ml/min. The mobile phase mixture was filtered through a 0.22-μm Millipore membrane, then degassed prior to use. The optimal fluorescence response for camptothecin was observed at excitation and emission wavelengths of 360 and 440 nm, respectively. Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.3. Animals

Adult, male Sprague–Dawley rats (280–320 g) were obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and were allowed to acclimate in their environmentally controlled quarters (24±1°C and 12:12 h light–dark cycle) for at least five days before experimentation. The rats were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and remained anesthetized continuously throughout the experimental period. The rats' body temperature was maintained at 37°C with a heating blanket.

2.4. Blood and brain microdialysis

The blood and brain microdialysis systems in this study consisted of a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and microdialysis

probes with dialyzing membranes. Flexible and rigid microdialysis probes were applied to sample the unbound endogenous or exogenous substances in rat blood and brain. A dialyzing membrane length of 10 mm with an outer diameter of 0.5 mm (CMA) was used for blood sampling while the brain microdialysis probe (CMA/12) had a membrane length of 3 mm and an outer diameter of 0.5 mm. The blood microdialysis probe was positioned within the jugular vein/right atrium and then perfused with ACD solution (citric acid 3.5 m*M*; sodium citrate 7.5 m*M*; dextrose 13.6 m*M*) at a flow-rate of 1 μl/min, by a microinjection pump (CMA/100). The rat was mounted on a Kopf stereotaxic frame for brain microdialysis. Its body temperature was maintained at 37°C with a heating pad. The brain microdialysis probe was perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{2+}; 4 m*M* K^+; pH 7.0) at a flow-rate of 1 μl/min. After washing with Ringer's solution, the microdialysis probe was implanted in the striatum (coordinates: AP 0.2 mm; ML −3.0 mm; DV −7.0 mm) according to the Paxinos and Watson atlas [14]. The positions of the probes were verified by standard histological procedure at the end of the experiments.

Outflows from blood or brain microdialysis probes were connected to an on-line injector (CMA/160) and automatically injected every 10 min. After dialysate levels had stabilized (approximately 2 h), then camptothecin (2 or 5 mg/kg) was intravenously administered via the femoral vein. From each sample 10 μl of dialysate was assayed using the microbore HPLC system.

2.5. Recovery of microdialysis probe

For in vivo recovery, the microdialysis probes were inserted into the rat blood or brain under sodium pentobarbital anesthesia. Ringer's solution containing camptothecin (100 ng/ml) was passed through the brain microdialysis probe at a constant flow-rate (1 μl/min) using an infusion pump (CMA/100) into the rat striatum. ACD solution containing camptothecin (100 ng/ml) was passed through the blood microdialysis probe at a constant flow-rate (1 μl/min) using an infusion pump (CMA/100) into the jugular vein. After a 2-h stabilization period subsequent to the probe implantation, the inlet (C_{in}) and outlet (C_{out}) concentrations of camptothecin were determined by HPLC. The in vivo recovery ratios ($Recovery_{in\ vivo}$) of camptothecin across a microdialysis probe in the blood and brain were calculated by the following equation [15,16]:

$$Recovery_{in\ vivo} = [(C_{in} - C_{out})/C_{in}] \cdot 100$$

2.6. Pharmacokinetics

Protein unbound camptothecin concentration data were obtained by correcting the microdialytic data for in vivo recovery of the respective microdialysis probes. Pharmacokinetic calculations of unbound camptothecin in rat the blood and brain were fitted to a biexponential ($C = Ae^{-\alpha t} + Be^{-\beta t}$) and monoexponential ($C = Ae^{-\alpha t}$) decay.

The distribution and elimination rate constants, α and β were calculated using the equation: α or $\beta = (\ln C_2 - \ln C_1)/(t_2 - t_1)$; where C_1 is the value of C at time t_1, and C_2 is the value of C at time t_2. Formation rate constants were calculated by extrapolation from the formation slope determined by the method of residuals. The areas under the concentration curves (AUCs) were calculated using the trapezoid method. Half-life ($t_{1/2}$) values were calculated using the equation: $t_{1/2,\alpha} = 0.693/\alpha$ and $t_{1/2,\beta} = 0.693/\beta$ for distribution and elimination half-life, respectively. The clearance (CL) was calculated as: CL = dose/AUC. The mean residence time (MRT) was calculated as MRT = AUMC/AUC, where AUMC is the area under the first moment–time curve. Volume of distribution (V_{dss}) was calculated by the method of Benet and Galeazzi [17]. Comparisons of pharmacokinetics data were performed using Student's *t*-test. Statistical significance was determined at the level of $P < 0.05$.

3. Results

Camptothecin in both blood and brain dialysates was adequately resolved using the validated microbore HPLC conditions [18]. The retention time of camptothecin was 6.2 min (Fig. 2). Fig. 2A shows a standard injection of camptothecin (5 ng/ml). Fig. 2B shows a chromatogram of a blank blood dialysate. None of the observed peaks interfered with

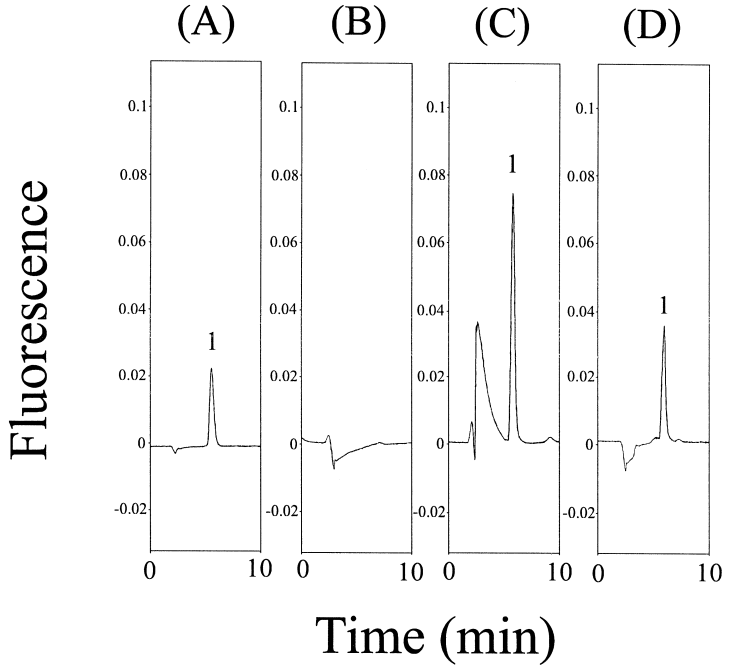

Fig. 2. Typical chromatograms of (A) a standard camptothecin (5 ng/ml), (B) a blank blood dialysate from the microdialysis probe before drug administration, and (C) a blood dialysate sample containing camptothecin (14.97 ng/ml) collected from a rat blood microdialysate 40 min post camptothecin administration (2 mg/kg, i.v.). (D) A brain dialysate sample containing camptothecin (6.84 ng/ml) collected from a rat brain microdialysate 30 min post camptothecin administration (5 mg/kg, i.v.). 1=Camptothecin.

the analysis of either compound. Fig. 2C shows a chromatogram of a blood dialysate sample containing camptothecin (14.97 ng/ml) collected from a rat blood microdialysate 40 min after camptothecin administration (2 mg/kg, i.v.). Fig. 2D shows a chromatogram of a brain dialysate sample containing camptothecin (6.84 ng/ml) collected from a rat brain microdialysate 30 min following camptothecin administration (5 mg/kg, i.v.).

The method used was linear ($r^2 > 0.995$) over a concentration range 5–500 ng/ml for camptothecin. The intra- and inter-day precision and accuracy of camptothecin fell well within the predefined limits of acceptability. All % bias and relative standard deviation (RSD) values were within $\pm 10\%$. The in vivo microdialytic recoveries of camptothecin (100 ng/ml) for blood and brain were $44.2 \pm 1.7\%$ and $15.0 \pm 0.3\%$, respectively using the retrograde method. This method is sufficiently sensitive to allow measurement of unbound camptothecin in rat blood and brain for the pharmacokinetic study. However, care must be taken to prevent obstruction of the microbore column during the course of the experiment.

Based on these microdialysis sampling data, the pharmacokinetics of unbound camptothecin in blood fitted best to a two-compartment model as follows: $C = 582e^{-0.093t} + 27e^{-0.012t}$ and $C = 677e^{-0.061t} + 34e^{-0.0062t}$ for camptothecin intravenous administration (2 and 5 mg/kg, respectively).

The pharmacokinetics of unbound camptothecin in the brain fitted the following one-compartment models: $C = 134e^{-0.076t}$ and $C = 422e^{-0.064t}$ for camptothecin intravenous administration (2 and 5 mg/kg, respectively). The pharmacokinetic parameters of camptothecin in the blood and brain are given in Table 1. Camptothecin seems to show a dose-related relationship between brain and blood concentrations. The average ratios of brain-to-blood AUCs in rats are 22% and 51% for 2 and 5 mg/kg of camptothecin administration, which indicate that camptothecin may transport into the brain with dose-

Table 1
Pharmacokinetic parameters of camptothecin in rat blood and brain following camptothecin administration (2 and 5 mg/kg, i.v., $n=6$) [data are expressed as mean±S.E.M. ($n=6$)]

Parameter	2 mg/kg	5 mg/kg
Blood		
$t_{1/2,\alpha}$ (min)	8.04±1.04	11.91±1.58[a]
$t_{1/2,\beta}$ (min)	66.92±15.88	137.55±19.44[a]
AUC (min μg/ml)	7.88±0.49	12.84±1.41[a]
Cl (l/kg/min)	0.26±0.016	0.41±0.038[a]
MRT (min)	28.67±5.1	52.01±14.64[a]
V_{dss} (l/kg)	7.70±1.62	22.39±7.21[a]
Brain		
$t_{1/2,\alpha}$ (min)	9.71±1.14	11.03±0.71
AUC (min μg/ml)	1.73±0.14	6.65±0.67[a]
MRT (min)	14.01±1.64	15.91±1.03

[a] The mean was significantly different from the lower (2 mg/kg) dose ($P<0.05$).

related manner. The average unbound brain/blood concentration ratio of camptothecin increased and reached the highest ratio at 20 and 30 min after camptothecin 2 and 5 mg/kg intravenous administration, respectively (Table 2).

4. Discussion

A previous report [19] has suggested that the pharmacokinetics of camptothecin derivative in the peripheral blood system appeared to fit a two-compartment model similar to the one described here.

Table 2
Unbound brain/blood concentration ratio of camptothecin after camptothecin administration (2 and 5 mg/kg, i.v.) [data are expressed as mean±S.E.M. ($n=6$)]

Time (min)	Unbound brain/blood concentration ratio	
	2 mg/kg	5 mg/kg
10	0.05±0.01	0.11±0.04
20	0.27±0.03	0.53±0.08
30	0.26±0.03	0.67±0.09
40	0.23±0.03	0.62±0.12
50	0.18±0.03	0.55±0.12
60	0.15±0.05	0.44±0.11
70	0.10±0.05	0.38±0.08
80		0.30±0.06
90		0.18±0.07
100		0.14±0.06

The ratio of the area under the concentration curve of brain over that in blood (AUC_{brain}/AUC_{blood}) was used as a measure of the drug penetration into the blood–brain barrier [20]. This steadily increasing ratio of further demonstrates that camptothecin penetrated the blood–brain barrier, which is in good agreement with findings by Blaney et al. [21], who showed that the camptothecin analog, 9-aminocamptothecin was capable of crossing the blood–brain barrier.

The present microdialysis technique provides protein-free samples that can be directly injected into a liquid chromatographic system for continuous in vivo monitoring of unbound drugs in biological samples such as blood or the brain. Further, this sampling method facilitates pharmacokinetic studies by reducing the effects of biological volume changes as compared to conventional biological fluid withdrawing assays [9]. It has been shown that the camptothecin (lactone form) is quite unstable, and the opening of the ring under basic conditions produces the water-soluble carboxylate form. To avoid the labile of camptothecin, an on-line microdialysis technique was used in this study.

Its potential for studying the pharmacokinetics of camptothecin in rat blood and brain are convincingly demonstrated here. Several in vivo techniques have been described for brain pharmacokinetic studies, including autoradiography, imaging methods [positron emission tomography (PET) and nuclear magnetic resonance (NMR)], cerebral fluid sampling, in vivo voltammetry, and intracerebral microdialysis. However, these techniques are too expensive for general laboratory use. Although intracerebral microdialysis has the disadvantage of being invasive, it is much cheaper than PET scanning or NMR and can be used in the general laboratory [9].

In this paper we showed the pharmacokinetics of unbound camptothecin in blood and brain after intravenous administration to rats. The fact that unbound camptothecin penetrates the blood–brain barrier is suggested to be the reason for its therapeutic value against brain tumors.

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