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Determination of unbound 20(*S*)-camptothecin in rat bile by on-line microdialysis coupled to microbore liquid chromatography with fluorescence detection

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Abstract

To evaluate the biliary excretion of unbound camptothecin, a flow-through microdialysis probe was constructed for bile sampling. The shunt linear probe was connected from the bile duct, between the liver side to the duodenum to avoid obstruction of the bile duct or bile salt waste. For automatic analysis of microdialysate, an on-line injector was connected to a microbore high-performance liquid chromatographic column with fluorescence detection. Samples were eluted with a mobile phase containing methanol–100 m*M* monosodium phosphoric acid (35:65, v/v, pH 2.5, adjusted with orthophosphoric acid). The limit of quantification was 1 ng/ml for camptothecin. Following camptothecin administration (5 mg/kg, i.v.), it was found in the bile microdialysate. It was concluded that the in vivo microdialysis technique yields useful data on the biliary excretion of camptothecin. This method is suitable for additional pharmacokinetic studies in rat bile.

Keywords: Camptothecin

1. Introduction

Camptothecin, an alkaloid of plant origin, is a well known inhibitor of topoisomerase I, and is also a very potent anticancer agent. Camptothecin is contained in a Chinese herb, *Camptotheca acuminata* [1], which is highly fluorescent and can be detected by fluorescence in nanogram levels. Several analytical methods have described camptothecin and its derivatives [2–6], but none have been used thus far in microdialysis and microbore high-performance liquid chromatography. Microdialysis sampling technique was originally developed for in vivo neurotransmitter release in the rat brain [7,8]. More recently, however, microdialysis sampling has extended its application into pharmacology and pharmacokinetics [9,10]. Over the past several years, microdialysis has been increasingly used for in vivo sampling of unbound endogenous or exogenous chemicals in the blood, brain or tissue etc. in various animal models [11–13]. Sampling by this technique involves continuous perfusion of fluid through microdialysis probes implanted into the tissue space being dialyzed. Published methods for the determination of camptothecin either a complex treatment of

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the biological samples [2–6] or are not suitable for automatic analysis.

In this study, we constructed a flow-through microdialysis probe [14,15], then inserted into the rat bile duct for sampling of camptothecin from the bile fluid following camptothecin was given intravenously. The dialysates were measured by an automatic microbore high-performance liquid chromatographic system with fluorescence detection. Microdialysis therefore appears to be a suitable technique for delivering a drug within a specific site, such as the bile duct to evaluate the biliary excretion of camptothecin.

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 de-ionized water from Millipore (Bedford, MA, USA) was used for all preparations.

2.2. Liquid chromatography

The liquid chromatographic system consisted of a chromatographic pump (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 System, West Lafayette, IN, USA) 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 [6]. Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

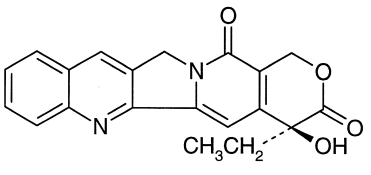

Fig. 1. Chemical structure of 20(*S*)-camptothecin.

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 acclimatize in their environmentally controlled quarters (24±1°C and 12:12 h light–dark cycle) for at least 5 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 rat's body temperature was maintained at 37°C with a heating blanket.

2.4. Microdialysis experiments

The bile duct microdialysis probes were constructed in house (Fig. 2) based largely on the design originally described by Scott and Lunte [14] and Hadwiger et al., [15]. A 7 cm dialysis membrane was inserted into polyethylene tubing (PE-60; 0.76 mm I.D.; 1.22 mm o.d., Clay-Adams, N.J., USA). The ends of the dialysis membrane and PE-60 were inserted into a silica tubing (40 μm i.d; 140 μm o.d., SGE, Australia) and PE-10 (0.28 mm I.D.; 0.61 mm o.d.), respectively. Both ends of tubing and the union were cemented with epoxy. Post bile duct cannulation, the rat was then perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{++}; 4 m*M* K^+; pH 7.0) by a microinjection pump (CMA 100) at flow-rate of 1 μl/min. Its body temperature was maintained at 37°C with a heating pad. Post dialysate level stabilization (approximately 2 h), the drug-free samples were collected, then camptothecin (5 mg/kg) was intravenously administered via the femoral vein in a

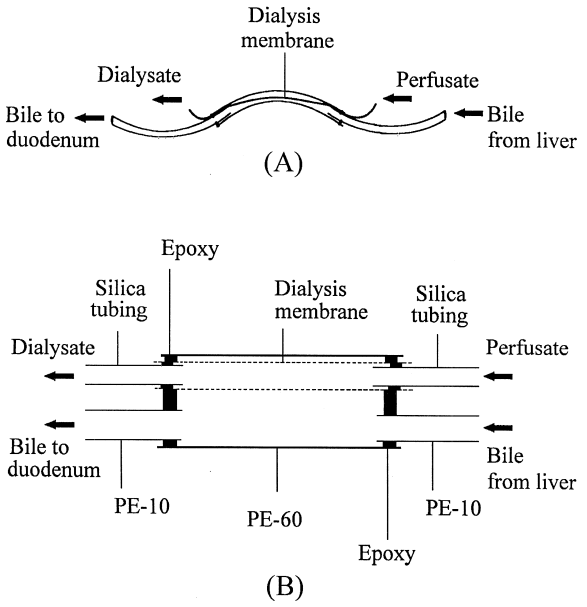

Fig. 2. Flow-through microdialysis probe used for rat bile sampling (A). Detailed description of a homemade bile microdialysis probe (B).

solution composed of 20% DMSO, 20% PEG 400, 30% ethanol, 30% Tween 80 [16]. Next each dialysate sample (10 μl) was assayed with the high-performance liquid chromatographic system. Ten μl of dialysate was collected every 10 min, then automatically assayed with the on-line HPLC system.

2.5. Recovery of microdialysis probe

For in vivo recovery, the bile microdialysis probes were inserted into the rat bile duct under sodium pentobarbital anesthesia. Ringer's solution containing camptothecin (100 ng/ml) was passed through the microdialysis probe at a constant flow-rate (1 μl/min) using an infusion pump (CMA-100). One hour after the probe implantation, which was a stabilization period, the inlet (C_{in}) and outlet (C_{out}) concentrations of camptothecin were determined by HPLC. The in vivo recovery ratio ($Recovery_{\text{in vivo}}$) of camptothecin across a microdialysis probe in the bile duct was calculated by the following equation [17,18]: $Recovery_{\text{in vivo}} = [(C_{in} - C_{out})/C_{in}] \times 100$.

2.6. Method validation

All calibration curves were required to have a correlation value of at least 0.995. The intra-day and inter-day variabilities were determined by quantitating six replicates at concentrations of 5, 10, 50, and 100 ng/ml using the HPLC method described above on the same day and six successive days, respectively. The accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentrations (C_{obs}) as follows: $accuracy(\%) = [(C_{nom} - C_{obs})/C_{nom}] \times 100$. The precision coefficient of variation (C.V.) was calculated from the observed concentrations as follows: $precision\ (\%) = [standard\ deviation\ (SD)/C_{obs}] \times 100$. The same data were used to determine both accuracy and precision.

3. Results and discussion

Acceptable chromatography defined in terms of peak shape and separation from endogenous interfering substances and sensitivity, were the primary considerations dictating the choice of the chromatographic system for the determination of unbound camptothecin in rat bile. To improve the sensitivity and resolution of camptothecin, a microbore column with fluorescence detection was used, under the conditions described above, the retention time of camptothecin was found to be 6.2 min (Fig. 3). Fig. 3A shows a standard injection of camptothecin (200 ng/ml). Fig. 3B shows a chromatogram of a blank blood dialysate. No peaks were observed that would interfere with the analysis of either compound. Fig. 3C shows a chromatogram of a bile dialysate sample containing camptothecin (311.68 ng/ml) obtained from a rat bile microdialysate 60 min post 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. Intra-day and inter-day precision and accuracy for camptothecin fell well within predefined limits of acceptability. All % bias and % C.V. values were within ±10% (Table 1).

Data concerning precision and accuracy of the results are presented in Table 1. Intra-day reproducibility was assessed by using six samples at four

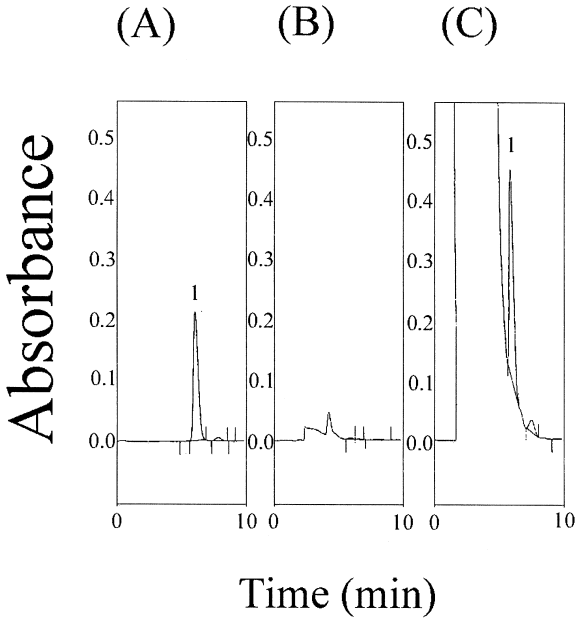

Fig. 3. Typical chromatograms of (A) a standard camptothecin (200 ng/ml), (B) a blank bile dialysate from the flow-through microdialysis probe pre-drug administration, and (C) a bile dialysate sample containing camptothecin (311.68 ng/ml) collected from a rat bile microdialysate 60 min post camptothecin administration (5 mg/kg, i.v.). 1: camptothecin.

Table 1
Intra-day and inter-day precision and accuracy of camptothecin determination ($n=6$)

Nominal concentration (ng/ml)	Observed concentration (ng/ml)[a]	C.V. (%)[a]	Accuracy (% Bias)[b]
Intra-assay			
5	5.2±0.4	7.2	4.0
10	9.9±0.3	3.0	−1.4
50	49.9±1.1	2.1	−0.2
100	100.1±0.5	0.5	0.1
Inter-assay			
5	5.4±0.3	4.9	7.2
10	9.8±0.3	2.9	−1.5
50	49.9±0.7	1.4	−0.3
100	100.4±0.6	0.6	0.4

[a] Observed concentration data are expressed as rounded means±SD.

[b] *Precision C.V.* (%) = [*standard deviation* (*SD*)/C_{obs}] × 100.

[c] *Accuracy* (%) = [(C_{nom} − C_{obs})/C_{nom}] × 100.

different concentrations of 5, 10, 50, and 100 ng/ml then, and analyzed on the same day. The coefficient of variations (C.V.s) were less than 8%. Day-to-day reproducibility was determined six times with three different quality control samples, within a one week period. The C.V.s at 5, 10, 50, and 100 ng/ml were less than 8%.

The in vivo microdialysis recovery of camptothecin was 87.12±1.56%, (based on 100 ng/ml standard of camptothecin). The concentrations vs. time of camptothecin in rat bile after camptothecin administration (5 mg/kg, i.v.) were calibrated by in vivo recovery and are shown in Fig. 4. The area under the concentration of unbound camptothecin was about 32.55±9.44 μg/ml/min (mean±S.E.M., $n=4$) calculated by trapezoidal rule. The samples were collected at 10 min intervals during the experimental course. As described above, this method is applicable to further pharmacokinetic studies on camptothecin in rat bile.

Several methods have been use for the measurement of camptothecin [2–6], but few are sufficiently sensitive or specific for used in bile fluid. This method has the advantage of allowing the measurement of unbound camptothecin in the automatic microdialysate of bile fluid, which might be more revealing than the study of protein bound camp-

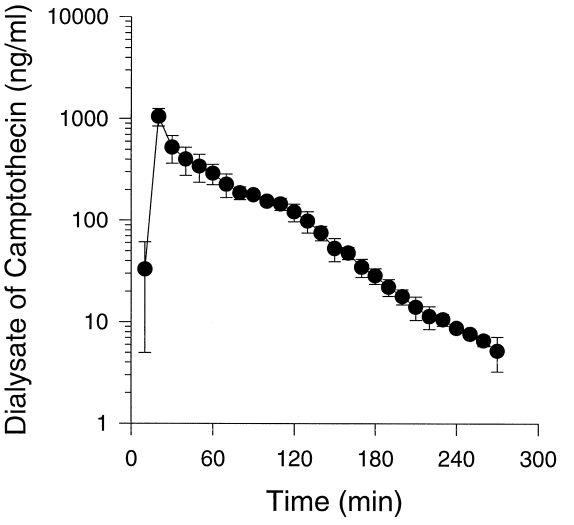

Fig. 4. Unbound dialysate of camptothecin in bile vs. time curves post camptothecin administration (5 mg/kg, i.v.). Error bars are expressed as means±S.E.M., $n=4$.

tothecin in the biological fluid. 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 [1,6,16]. To avoid the labile of camptothecin, an on-line microdialysis technique was used in this study.

This method is sufficiently sensitive to allow measurement of unbound camptothecin in rat bile. However, care must be taken to prevent obstruction of the microbore column during the course of experiment.

This microdialysis technique provides protein-free samples that can be directly injected onto a liquid chromatographic system for continuous in vivo monitoring of unbound drugs in bile. Further, this sampling method facilitates pharmacokinetic studies, which reduce the effects of biological fluid volume changes as compared to the conventional bile withdrawing assay method. Its potential for studying the pharmacokinetics of camptothecin in rat bile is convincingly demonstrated in this experiment.

Other methods using bile fluid collection to measure drug concentration in the bile fluid have been described elsewhere [19]. However, this method requires a complicated step for sample clean up. By applying the microdialysis technique to biliary excretion study, the total number of animals needed can be substantially reduced because the technique involves a sampling technique which does not withdraw body fluid and so does not disturb body homeostasis.

In conclusion, we have constructed a successful flow-through microdialysis probe and measured the concentration accurately of unbound camptothecin in the rat bile by microdialysis. Measurement of camptothecin in bile fluid may be of interest in the investigation of the mechanism of active transport of drug or its metabolite from blood to bile.

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