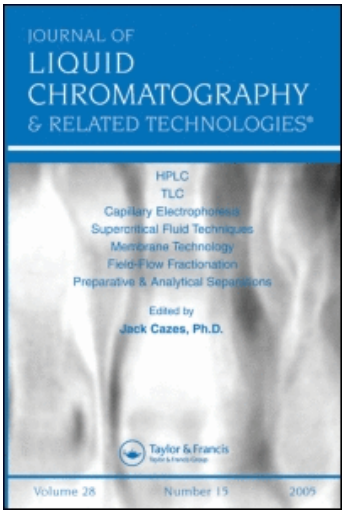

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BILIARY EXCRETION OF GRANISETRON BY MICRODIALYSIS COUPLED TO HPLC WITH FLUORESCENCE DETECTION

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ABSTRACT

Recent studies have shown that granisetron incurs glucuronide conjugation in the rat. This metabolic reaction may facilitate biliary excretion and reabsorption of the drug through enterohepatic circulation. This study was designed to evaluate the biliary excretion of granisetron employing a specially designed microdialysis shunt probe to monitor biliary drug concentrations without fluid losses. To avoid obstruction of the bile duct or bile salt waste, the shunt linear probe connected the bile duct between liver and duodenum. The utility of this microdialysis design was demonstrated by studying the hepatic metabolism and biliary excretion of granisetron in the rats. Following intravenous administration (3 mg/kg), granisetron was measurable in the bile microdialysate. Samples were eluted with a mobile phase containing 25 mM acetate buffer (pH 5.3): acetonitrile (78:22, v/v). The 25 mM acetate buffer was prepared with 25 mM sodium acetate with pH adjusted to 5.3 by glacial acetic acid. It was concluded that the in vivo microdialysis technique yielded useful data on the biliary excretion of granisetron.

INTRODUCTION

Microdialysis sampling technique was originally developed for the in vivo neurotransmitter release in the brain[1,2] but has since been extended to pharmacological and pharmacokinetics studies.[3,4] Over the past several years, microdialysis has been increasingly used for in vivo sampling of unbound endogenous or exogenous compounds in the blood, brain, or tissue, etc. in various animal experiments.[5-7] Sampling by this technique involves continuous perfusion of fluid through microdialysis probes implanted in the tissue space being dialyzed.

In theory, a microdialysis probe is made up of a semi-permeable dialysis membrane, which allows passive diffusion. For the purpose of drug administration or sampling, low molecular weight substances diffuse along the concentration gradient toward or away from the probe perfused with an appropriate solution at a constant rate by a perfusion pump.[8,9] Prior to the advent of microdialysis, attempts to determine drug concentration continuously from the bile duct with no bile loss had limited success.

To investigate the biliary excretion of granisetron, we designed a flow-through microdialysis probe[10,11] and inserted it into the rat bile duct for the sampling of granisetron from bile fluid following intravenous granisetron administration. Granisetron in the dialysate samples were measured by a high-performance liquid chromatographic method with fluorescence detection.[12] The results indicated that microdialysis appeared to be a suitable technique for sampling a drug within a specific site, such as bile duct, for the computation of pharmacokinetic parameters.

EXPERIMENTAL

Chemicals and Reagents

Granisetron hydrochloride was purchased from SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, UK). The chromatographic reagent and gradient grade solvents were obtained from E. Merck (Darmstadt, Germany). Triple de-ionized water (Millipore, Bedford, MA, USA) was used for all preparations.

Chromatography

The chromatographic system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), a Rheodyne Model 7125 injector equipped with a 20 μL sample loop and a fluorescence detector (Waters 474 scanning fluorescence detector, Milford, MA, USA). Such sample was separated using a

reversed-phase C18 column (150 x 4.6 mm; 5 μm; Cosmosil, Kyoto, Japan). Chromatography was performed at ambient temperature. The mobile phase consisted of 25 mM acetate buffer (pH 5.3) : acetonitrile (78:22, v/v). The 25 mM acetate buffer was prepared with 25 mM sodium acetate and pH was adjusted to 5.3 by glacial acetic acid. The flow rate was 1 mL/min. The mobile phase was filtered with a 0.45 μm Millipore membrane prior to being used for elution. The excitation and emission wavelengths for optimal fluorescence response for granisetron had been determined to be 305 and 360 nm respectively. Output data from the fluorescence were amplified and integrated on an integrator (SCI Chromatocorder 12, Tokyo, Japan).

Microdialysis Experiment

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 specific-pathogens-free and kept in environmentally controlled quarters (24 ± 1°C and 12:12 h light-dark cycle). The rats were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and anesthesia was maintained throughout the experiment period.

The bile duct microdialysis probes were constructed in house. A 7-cm dialysis membrane was inserted into polyethylene tubing (PE-60; 0.76 mm i.d.; 1.22 mm o.d.). The ends of dialysis membrane and the PE-60 tubing were connected to a silica tubing (40 μm i.d; 140 μm o.d., SGE, Australia) and a PE-10 tubing (0.28 mm i.d.; 0.61 mm o.d.), respectively. Both ends of tubing and the union were cemented with epoxy.[10,11] Following bile duct cannulation, the microdialysis probe was perfused with Ringer's solution (147 mM Na^+; 2.2 mM Ca^{++}; 4 mM K^+; pH 7.0). Body temperature of the rat was maintained at 37 °C with a heating pad. After dialysate levels had stabilized (approximately 2 h), the drug-free reference samples were collected and then granisetron (3 mg/kg) was intravenously administered via the femoral vein. Granisetron in the dialysate samples (20 μL) was assayed with the high performance liquid chromatographic system. The microsyringe and the 20 μL loop of injector were washed with methanol before and between sample injections.

Recovery

For in vivo recovery, the bile microdialysis probes were inserted into the rat bile duct under anesthesia with sodium pentobarbital. Ringer's solution containing granisetron (100, 200, or 500 ng/mL) was passed through the microdialysis probe at a constant flow rate (2 μL/min) using an infusion pump (CMA-100). One hour was allowed after the probe implantation as a stabilization period, the inlet (C_{in}) and outlet (C_{out}) concentrations of granisetron

were then determined by HPLC. The in vivo recovery ratio ($Recovery_{in\ vivo}$) of granisetron across a microdialysis probe in bile duct was calculated by the following equation:[13,14]

$$Recovery_{in\ vivo} = (C_{in} - C_{out}/C_{in})$$

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 20, 50, 100, 200, and 500 ng/mL using the HPLC method described above on the same day and four different 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}] x 100.

The precision coefficient of variation (C.V.) was calculated from the observed concentrations as follows: precision (%) = [standard deviation (S.D.) / C_{obs}] x 100.

The same data were used to determine both accuracy and precision.

Pharmacokinetic Analysis

Calculated pharmacokinetic values were obtained by treatment of individual sets of data. Unbound granisetron data in bile were fitted to biexponential decay. The elimination rate constant, β was calculated using the equation:

$\beta = (\ln C_1 - \ln C_2) / (t_2 - t_1)$, where C_1 and C_2 were the concentrations at times t_1 and t_2, respectively.

Formation rate constants were calculated from the extrapolated formation slope determined by the method of residuals. The areas under the concentration curves (AUCs) were calculated by the trapezoidal rule and extrapolated to time infinity by the addition of $AUC_{t\text{-}inf} = C_t/\beta$. The AUC values for bile were thus given by the sum of the products of the measured concentrations and the collection time interval, plus the residual area, that is: $AUC = AUC_{0\text{-}t} + AUC_{t\text{-}inf}$. Elimination half-life ($t_{1/2,.}$) value was calculated using the equation:

$$t_{1/2,.} = 0.693 /\beta$$

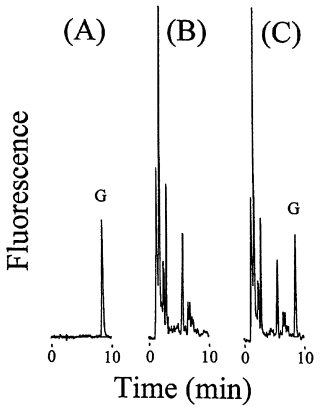

Figure 1. Typical chromatograms of (A) a standard granisetron (100 ng/mL), (B) a blank bile dialysate from the flow-through microdialysis probe before drug administration, and (C) a bile dialysate sample containing granisetron (81.55 ng/mL) collected from a rat bile microdialysate 12 min after granisetron administration (3 mg/kg, i.v.). G: granisetron.

RESULTS AND DISCUSSION

The liquid chromatographic system was applied to determine granisetron from rat bile duct. Under the conditions described above, the HPLC retention times of granisetron was found to be 10.3 min (Figure 1). Figure 1A shows a standard injection of granisetron (100 ng/mL). Figure 1B shows a chromatogram of a blank bile dialysate sample obtained from bile duct microdialysis before drug administration. Figure 1C shows a chromatogram of a bile dialysate sample obtained from bile duct microdialysis 60 min after granisetron administration (3 mg/kg, i.v.).

To optimize the granisetron separation from the dialysate of bile fluid, acetonitrile concentration and pH value of buffer solution were modified from conditions described in previous report.[12]

Data concerning precision and accuracy of the results are presented in Table 1. Intra-day reproducibility was assessed by using six samples at four different concentrations (20, 50, 100, and 200 ng/mL) and analyzed on the same day. The coefficient of variations (C.V.s) were less than 3%.

Day-to-day reproducibility was determined six times with three different quality control samples, within one week. The C.V.s at 20, 50, 100, and 200 ng/mL were less than 6%.

Table 1

Precision and Accuracy of Granisetron[a]

Nominal Concn.	Measured Concn.[b]	C.V. (%)	Bias (%)
Intra-Assay			
20	19.95 ± 0.49	2.45	-0.25
50	50.55 ± 0.88	1.70	1.09
100	100.98 ± 1.53	1.52	0.98
200	200.05 ± 1.15	0.58	0.02
Inter-Assay			
20	19.77 ± 1.11	5.61	-1.15
50	50.42 ± 2.17	4.29	0.84
100	100.59 ± 1.62	1.61	0.59
200	199.93 ± 1.62	0.81	-0.03

[a] (ng/mL). [b] Data are expressed as means ± S.E.M. (n=6).

Table 2

In Vivo Recovery of Granisetron for Bile Microdialysis Probe*

Granisetron Concn. (ng/mL)	In Vivo (%)
100	63.56 ± 2.74
200	61.27 ± 2.29
500	60.99 ± 3.35

[a] Data are expressed as means ± S.E.M. (n=6).

The in vivo recovery of granisetron at the concentrations of 100, 200, and 500 ng/mL did not show any significance differences. The average recovery ranges between about 60-64% (Table 2). The linear range of granisetron in the method was 20-500 ng/mL.

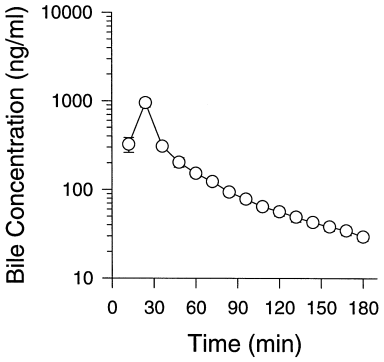

Figure 2. Bile concentration of granisetron versus time curves after granisetron administration (3 mg/kg, i.v.). Error bars are means±S.E.M. (n=4).

The linear relationship was observed with an equation of the regression line:

$y = 0.04\ x - 0.35$ ($r^2 = 0.995$, $n = 10$).

The concentration versus time curve is shown in Figure 2, and the pharmacokinetic results reflect the fact that the disposition of granisetron in rat bile appeared to fit a two elimination phase model after 20 min of granisetron administration (3 mg/kg, i.v.). The elimination half-life was about 51.07 ± 0.75 min (mean ± S.E.M., n=4), and the AUC of granisetron was about 287.76 ± 75.13 μg/mL/min (mean ± S.E.M., n=4). The AUC of granisetron in bile was about fifty folds higher than blood level. The results suggest that granisetron may be actively transported from blood to bile against a concentration gradient.

Drug diffusion through the dialysis membrane depends on the molecular mass, configuration, and ionic charge of the molecule.[15,16] The concentration of the compound in the dialysate represents only a percentage of the original concentration in the tissue compartment being dialysed. The ratio between the original concentration and the concentration in the dialysate is the recovery rate. To minimize the recovery difference between in vitro and in vivo, a retrograde in vivo recovery was used in the study. The extra length in the bile dialysis membrane may account for the fact that the recovery rate of granisetron in the bile duct was much higher than those in the blood and brain.[12]

Other methods used to measure drug concentration in the bile fluid have been described using bile fluid collection.[17] However, such samples may have to go through a relatively complicated process of sample cleanup before they can be analyzed.

By applying the microdialysis technique to biliary excretion study the number of animals needed can be substantially reduced, because the technique involves a sampling technique which does not incur body fluid losses and therefore does not disturb blood homeostasis.

In conclusion, the method has demonstrated that microdialysis, coupled to appropriate liquid chromatography with fluorescence detection can be applied to rapidly, accurately, and precisely determine granisetron in rat bile. Results using such techniques provided proof that the granisetron was excreted in the bile.

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