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Determination of unbound theophylline in rat blood and brain by microdialysis and liquid chromatography

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Abstract

To investigate the mechanism by which theophylline crosses the blood–brain barrier (BBB) and its disposition, we determined unbound theophylline in rat blood and brain using microbore liquid chromatography coupled with microdialysis. Microdialysis probes were inserted into the jugular vein and the brain striatum of male Sprague–Dawley rats. Then theophylline at dosage of 10 or 30 mg/kg was administered through the femoral vein. Theophylline and dialysates were separated using a microbore phenyl–hexyl column (150 mm × 1 mm, 5 μm). The mobile phase comprised of acetonitrile–methanol–10 mM monosodium phosphate (pH 3.0) (10:20:70, v/v/v). The UV wavelength was set at 270 nm. The concentration-response relationship was linear over a concentration range of 0.05–50 μg/ml; intra-assay and inter-assay precision and accuracy of theophylline fell within 10%. Average in vivo recoveries were 0.74 ± 0.06 in blood and 0.27 ± 0.07 in brain with theophylline at concentrations 1, 2 and 5 μg/ml. This biological sampling method thereby allowed the determination of theophylline levels in blood and brain tissues. The disposition of theophylline in the blood and brain tissue suggests that there was rapid exchange and equilibration between the blood and brain system. The drug–drug interaction results indicate that theophylline was able to cross BBB, but that it might not be regulated by *p*-glycoprotein to the pharmacokinetics of theophylline.
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Keywords: Microdialysis; Theophylline; Cyclosporine

1. Introduction

Theophylline is a methylxanthine which acts as a bronchial smooth muscle relaxant and a suppressor of non-bronchodilator response of airways, and whose bronchodilator mechanism is through the inhibition of phosphodiesterase by increase of cAMP. Because of serious morbidity and mortality of theophylline toxicity, monitoring the plasma concentrations of theophylline to decrease its side effects has been recommended. Theophylline was reported [1] to be metabolized by multiple forms of cytochrome P450s in human, rabbit, and rat liver microsomes. These metabolites are 1-methylxanthine, 3-methylxanthine, 1-methyluric acid and 1,3-dimethyluric acid [2].

It is also well known that toxic doses of theophylline may cause effects in the central nervous system (CNS) such as stupor and convulsions [3]. *p*-Glycoprotein is a membrane-bound ATP dependent efflux transporter. It is becoming increasingly recognized that *p*-glycoprotein can have significant effects on the absorption, distribution, metabolism and excretion of drugs. These effects are particularly apparent when considering the oral bioavailability and the CNS distribution of several therapeutic compounds [4]. It is not known whether theophylline may also be a substrate for *p*-glycoprotein.

Most of the liquid chromatographic (LC)-based approaches reported to date result in the measurement of total drug concentration rather than the unbound fraction. Furthermore, some studies have attempted to monitor the protein-unbound form of theophylline, focusing on the level in blood and brain [5,6]. *p*-Glycoprotein is expressed in normal tissues with excretory functions, such as liver, kidney, intestine, blood–brain barrier (BBB), etc. which potentially results in drug–drug interaction. This method causes minimal disturbance to the hemodynamics and physiological processes of the experimental animal. The data thus obtained provide the basis for the construction of pharmacokinetic profiles and analyses.

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Although several LC methods have been described for the measurement of theophylline from biological sample [1–3] and summarized in a table [7], few assays has been reported to measure protein unbound theophylline in rat blood and brain. In 2000, Kanazawa et al. used a column-switching method coupled with atmospheric pressure chemical ionization (LC–APCI-MS) to determine theophylline and its metabolites in biological samples [8]. Here in order to investigate the BBB penetration of theophylline and its distribution ratio, we developed a microdialysis technique coupled with sensitive microbore liquid chromatographic system to monitor the protein-unbound theophylline level in rat blood and brain. In addition, cyclosporine, a *p*-glycoprotein inhibitor, was co-administered with theophylline to explore the mechanism of the pharmacokinetics of theophylline in rats.

2. Experimental

2.1. Chemicals and reagents

Theophylline and cyclosporine (Sandimmun) were purchased from Sigma (St. Louis, MO, USA) and Novartis (Basle, Switzerland), respectively. Chromatographic solvents were obtained from E. Merck (Darmstadt, Germany). Triple deionized water from Millipore (Bedford, MA, USA) was utilized for all preparations.

2.2. Liquid chromatography

HPLC was performed with a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), a Rheodyne Model 7125 injector equipped with a 10 μl sampling loop and an ultraviolet detector with micro flow cell (Varian, Walnut Creek, CA, USA). Separation was achieved by a Phenomenex Luna microbore phenyl–hexyl column (150 mm × 1 mm i.d.; particle size 5 μm, Torrance, CA, USA). The mobile phase consisted of acetonitrile–methanol–10 mM monosodium phosphate (pH 3.0) (10:20:70, v/v/v), with a flow-rate of 0.05 ml/min, and the wavelength was 270 nm. Output data from the detector were integrated using an EZChrom chromatographic data system (Scientific Software, San Roman, CA, USA).

2.3. Assay validation

Quantification was obtained by the measurement of drug concentration against the peak area of theophylline. The concentration of unknown dialysate samples was determined by using the linear regression line (unweighted) of the concentration standard versus peak area. The precision of the method was expressed as the intra-day and inter-day coefficients of variation (%), which were assayed (six replicates) at concentrations of 0.05, 0.1, 0.5, 1, 5, 10 and 50 μg/ml on the same day and on 6 sequential days, respectively. The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of the observed concentration (C_{obs}) as follows: bias (%) = $[(C_{obs} - C_{nom})/(C_{nom})] \times 100$. The R.S.D. was calculated from the observed concentrations as follows [9]: precision (% R.S.D.) = $[\text{S.D.}/C_{obs}] \times 100$.

2.4. Animals

The institutional animal experimentation committee of the National Research Institute of Chinese Medicine reviewed and approved all experimental protocols involving animals. Male, specific pathogen-free Sprague–Dawley rats were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei. The animals had free access to food (Laboratory rodent diet No. 5P14, PMI Feeds, Richmond, IN, USA) and water until 18 h prior to being supplied for experiments, when only food was removed. The rats were initially anaesthetized with urethane 1 g/ml and α-chloralose 0.1 g/ml (1 ml/kg, i.p.), and remained anaesthetized throughout the experimental period. The rats' body temperature was maintained at 37 °C with a heating pad during the experiment.

2.5. Blood and brain microdialysis

Blood and brain microdialysis systems comprised of a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and microdialysis probes. The dialysis probes for blood (10 mm in length), and brain (3 mm in length) were made of silica capillary in a concentric design [10–12]. Their tips were covered by dialysis membrane (Spectrum Lab., 200 μm i.d. with a cut-off at nominal molecular weight of 13,000, Laguna Hills, CA, USA) and all unions were cemented with epoxy. At least 24 h was allowed for the epoxy to dry. The blood microdialysis probe was located within the jugular vein/right atrium and then perfused with anticoagulant dextrose (ACD) solution (citric acid 3.5 mM; sodium citrate 7.5 mM; dextrose 13.6 mM) at a flow rate of 2 μl/min employing the microinjection pump. For brain microdialysis, the microdialysis probe was implanted in the right striatum (coordinates: AP 0.4 mm, ML −3.0 mm, DV −7.0 mm) according to the Paxinos and Watson [13]. The microdialysis probe was perfused with Ringer's solution (147 mM Na^+; 2.2 mM Ca^{2+}; 4 mM K^+; pH 7.0) at a flow-rate of 2 μl/min. The positions of the probes were verified by standard histological procedure at the end of experiments.

A retrograde calibration technique was utilized during in vivo recovery. The blood and brain microdialysis probes were inserted into the jugular vein of rats, and brain striatum under anesthesia. Following a stabilization period of 2 h post probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of theophylline were determined by HPLC. ACD solution containing theophylline (for blood microdialysis) or Ringer's solution containing theophylline (for brain microdialysis) was perfused through the probe at a constant flow rate (2 μl/min) by the infusion pump (CMA/100). The

in vivo relative recovery (R_{dial}) of theophylline across the microdialysis probe was calculated by the following equation: $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$. The microdialysate recovery and concentration calculations were performed according to our previous reports [12,14]. Theophylline microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows: $C_u = C_m/R_{dial}$.

2.6. Drug administration

The drug was subsequently administered according to the following study design. Six animals were used in each group. All the administered drugs were dissolved in saline with injection volume 1 ml/kg by i.v. bolus injection. The control group received a dose of 10 mg/kg theophylline. For the cyclosporine treated group, cyclosporine 10 mg/kg was injected via the femoral vein 10 min before theophylline 10 mg/kg injection, respectively. Outflow dialysates from blood, and brain were collected in a fraction collector (CMA/140) every 10 min. These dialysate samples were measured by HPLC during the same experimental day.

2.7. Pharmacokinetics

Pharmacokinetic calculations were performed on each individual animal's data utilizing the pharmacokinetic calculation software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA) by a non-compartmental method. The area under the concentration–time curve (AUC), the area under the first moment curve (AUMC) and the mean residence time (MRT) were all calculated by statistical moments [15]. Formation rate constants were calculated from the extrapolated formation slope determined by the residual method. The AUCs from time zero to time infinity ($AUC_{0-\infty}$) were calculated by the trapezoidal rule and extrapolated to time-infinity by the addition of $AUC_{t-\infty}$. An analogous method was employed to calculate the AUMC with the concentration versus time data, as follows:

$$\mathrm{AUC} = \mathrm{AUC}_{0-t} + \mathrm{AUC}_{t-\infty} = \mathrm{AUC}_{0-t} + \frac{C_{\mathrm{last}}}{\lambda_z}$$

$$\mathrm{AUMC} = \mathrm{AUMC}_{\mathrm{last}} + \left(t_{\mathrm{last}} \times \frac{C_{\mathrm{last}}}{\lambda_z}\right) + \frac{C_{\mathrm{last}}}{(\lambda_z)^2}$$

where C_{last} and t_{last} are the last observed concentration and time, respectively; and λ_z the terminal slope which is estimated by linear regression of the logarithmic value of the last observed data. The clearance (Cl) and MRT were calculated as follows: Cl = dose/AUC and MRT = AUMC/AUC. The blood-to-brain distribution was calculated as follows: AUC_{brain}/AUC_{blood}.

2.8. Statistical analysis

The statistical analysis was performed with SPSS version 10.0 (SPSS Inc. Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for the comparison between the control (theophylline alone) and cyclosporine treated group. All statistical tests were performed to the two-sided 5% level of significance.

3. Results and discussion

3.1. Chromatography

Typical chromatograms of theophylline in rat blood and brain are shown in Figs. 1 and 2, respectively. The retention time of theophylline was 5.1 min. The calibration graph of theophylline was obtained prior to LC analysis of dialysates over concentration range of 0.05–50 μg/ml. None of the

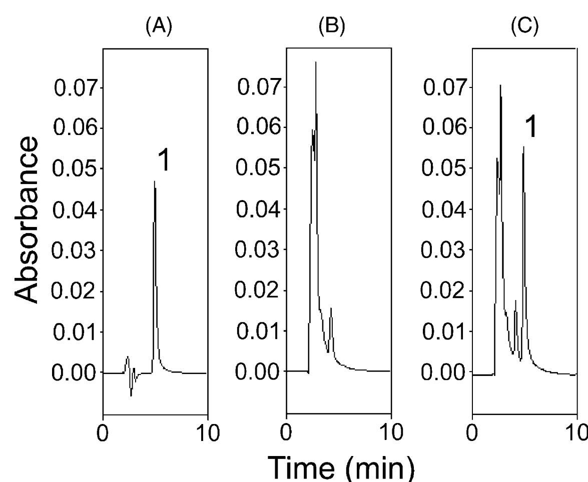

Fig. 1. Typical chromatograms of (A) standard theophylline (5 μg/ml), (B) blank blood dialysate from the microdialysis probe before drug administration, and (C) blood dialysate sample containing theophylline (6.35 μg/ml) collected from the rat blood microdialysate 20 min after theophylline administration (10 mg/kg, i.v.). (1) Theophylline.

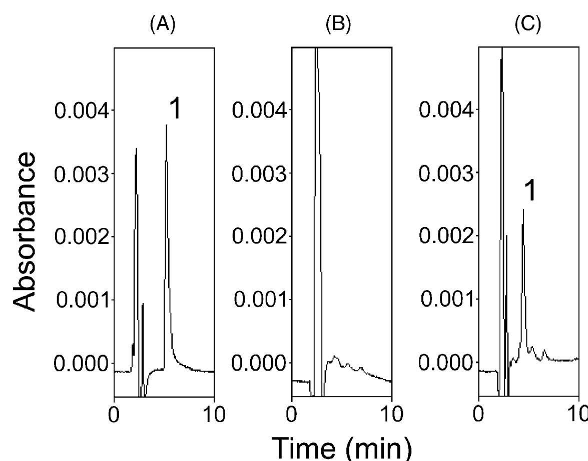

Fig. 2. Typical chromatograms of (A) standard theophylline (0.5 μg/ml), (B) blank brain dialysate from the flow-through microdialysis probe before drug administration, and (C) brain dialysate sample containing theophylline (0.2 μg/ml) collected from the rat brain microdialysate 20 min after theophylline administration (10 mg/kg, i.v.). (1) Theophylline.

Table 1
Intra-day and inter-day precision (R.S.D.) and accuracy (bias) of the HPLC method for the determination of theophylline[a]

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	R.S.D. (%)	Bias (%)
Intra-day			
0.05	0.045 ± 0.004	8.9	−10.0
0.10	0.102 ± 0.002	2.0	2.0
0.50	0.508 ± 0.014	2.8	1.6
1.00	0.996 ± 0.007	0.7	−0.4
5.00	4.99 ± 0.16	3.2	−0.3
10.00	9.92 ± 0.28	2.8	−0.8
50.00	50.68 ± 0.93	1.8	1.4
Inter-day			
0.05	0.049 ± 0.004	8.2	−2.0
0.10	0.103 ± 0.002	1.9	3.0
0.50	0.498 ± 0.012	2.4	−0.4
1.00	1.001 ± 0.005	0.5	0.1
5.00	5.18 ± 0.25	4.8	3.5
10.00	10.08 ± 0.19	1.9	0.9
50.00	48.46 ± 0.46	0.9	−3.1

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

observed peaks interfered with the analyte in the chromatogram of the blood and brain samples. The limits of detection (LOD) and quantification (LOQ) were 0.01 and 0.05 μg/ml, respectively. In this experiment, the performance of microbore phenyl column was compared to the conventional ODS column, the peak shape, peak tailing and shorter run time have been improved [16]. The phenyl-based microbore column for the separation of theophylline from biological dialysates has not been reported.

3.2. Determination of theophylline

This chromatographic system was validated for both intra- and inter-day precision (0.7–8.9%) and accuracy (0.3–10.0%) (Table 1). Accuracy and precision values within ±10% covering the actual range of experimental concentrations were considered acceptable [9]. Average in vivo recovery levels of theophylline were 0.74 ± 0.06 in blood and 0.27 ± 0.07 in brain with theophylline at concentrations 1, 2 and 5 μg/ml

Table 2
In vivo microdialysate recovery of theophylline in rat blood and brain

Concentration (μg/ml)	Recovery
Blood	
1	0.77 ± 0.07
2	0.74 ± 0.04
5	0.72 ± 0.05
Average	0.74 ± 0.06
Brain	
1	0.30 ± 0.09
2	0.27 ± 0.07
5	0.24 + 0.05
Average	0.27 ± 0.07

Data are expressed as means ± S.D.($n = 4$).

(Table 2). The transport properties of the microdialysis probe are described by the extraction efficiency or the term of recovery. Slow perfusion rate obtains higher recovery but smaller sample volume per unit time. Actually, several factors are interrelated to the recovery, such as sample volume, proteins surrounding the dialysis membrane, perfusion rate and sampling interval. The in vivo protein binding of theophylline in rat blood has been reported of 48.8 ± 6.2% [17].

3.3. Pharmacokinetic application

These pharmacokinetic curves indicate that the disposition of theophylline in rat brain exhibited a peak concentration after 20–30 min of theophylline administration (10 and 30 mg/kg), followed by a slow elimination phase. The distribution ratio of theophylline in brain and blood (AUC_{brain}/AUC_{blood}) were 0.05 ± 0.01, and 0.10 ± 0.01, for the doses of 10 and 30 mg/kg, respectively (Table 3).

Our results indicating the efflux permeability of theophylline through the BBB were evaluated using in vivo brain microdialysis technique, which is in agreement with the previous report [18,19]. Theophylline was rapidly distributed into the brain at a peak time of 20–30 min. Furthermore, administration of higher dose of theophylline 30 mg/kg produce an approximately two-fold increase in the brain distri-

Table 3
Pharmacokinetic parameters of the control group, with theophylline administration (10 or 30 mg/kg, i.v.); and the treated group, with cyclosporine (Cys) 10 mg/kg injected via femoral vein 10 min prior to theophylline 10 or 30 mg/kg administration.

Parameters	Theophylline 10 mg/kg		Theophylline 30 mg/kg	
	Without Cys	With Cys	Without Cys	With Cys
Blood				
AUC (min μg ml^{-1})	1596 ± 253	1276 ± 152	6468 ± 639	5930 ± 587
Cl (l/(kg min))	6.98 ± 1.14	8.27 ± 0.92	4.90 ± 0.56	5.33 ± 0.68
MRT (min)	163 ± 35	174 ± 16	265 ± 33	212 ± 14
Brain				
AUC (min μg ml^{-1})	71 ± 7	72 ± 8	638 ± 76	688 ± 63
MRT (min)	130 ± 8	104 ± 10	144 ± 4	135 ± 8
AUC_{brain}/AUC_{blood}	0.05 ± 0.01	0.05 ± 0.01	0.10 ± 0.01	0.12 ± 0.01

Data are expressed as mean + S.E. ($n = 6$).

bution ratio (AUC_{brain}/AUC_{blood}), compared with administration of lower dose of theophylline 10 mg/kg. The distribution ratio of theophylline through the BBB appears in a dose related manner.

In order to investigate the mechanisms of *p*-glycoprotein related BBB penetration of theophylline, we focused on drug–drug interaction of theophylline and cyclosporine (a *p*-glycoprotein inhibitor). Our results indicate that the distribution ratio of theophylline from blood to brain (AUC_{brain}/AUC_{blood}) in the cyclosporine 10 mg/kg co-administered groups were not significantly different from the theophylline-alone groups for the dosages of 10 and 30 mg/kg, respectively (Table 3). These results imply that the BBB penetration of theophylline might not be regulated by the *p*-glycoprotein.

In conclusion, a rapid and sensitive microbore liquid chromatographic system for the determination of unbound theophylline in rat blood and brain was developed. This method exhibits no endogenous interference and its sensitivity is sufficient for the measurement of protein-unbound theophylline in blood and brain. Theophylline passes through the BBB, but it might not be regulated to *p*-glycoprotein transported system.

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