Research Article

Pharmacokinetics of Acetaminophen in the Hypothalamus of Rats Based on *in vivo* Microdialysis

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Abstract

To explore the studying method for pharmacokinetics in the target site of drugs, the pharmacokinetic process of acetaminophen in the hypothalamus of rats was investigated. Male Sprague-Dawleyrats were anaesthetized and secured in a stereotaxic frame. A microdialysis probe was implanted into the hypothalamus and perfused with artificial cerebrospinal fluid at a flow rate of 2 μL/min. Adaptation for 1 h, rats were administrated with acetaminophen (150 mg/kg, i.p.) and microdialysates were collected continuously at 12-min intervals for 6 h. The acetaminophen concentrations in microdialysates were determined by HPLC-Ultraviolet detection (HPLC-UV), and the concentration-time profile and pharmacokinetic parameters of acetaminophen were calculated by DAS software. The results showed that the concentration-time curve of acetaminophen in the hypothalamus of rats was fitted to a one-compartment open model. The main pharmacokinetic parameters $t_{1/2}$, T_{max}, C_{max} and AUC_{inf} were (1.95 ± 0.59) h, (1.26 ± 0.22) h, (11.39 ± 2.17) μg/mL and (58.04 ± 18.39) μg·h/mL, respectively. In conclusion, by means of *in vivo* microdialysis approach, the pharmacokinetic process of acetaminophen in the hypothalamus of rats is investigated and an experimental method for studying pharmacokinetics of drugs in the target site is established, which is simple, feasible and reliable.

Keywords: Acetaminophen, HPLC, in vivo Microdialysis, Pharmacokinetics, Rats

Introduction

In pharmacokinetic studies, the traditional method is to measure drugs or their metabolites concentrations in blood, and calculate pharmacokinetic parameters based on the plasma drug and/or its metabolite concentrations, which are used for guiding drug administration and dosing regimens in clinic. However, the biochemical events and pharmacological effects do not usually take place in the bloodstream, but in target organs and/or tissues [1, 2]. Therefore, it is unreasonable to use the plasma drug or its metabolite concentration to in place of the target organ/tissue drug concentration for calculating pharmacokinetic parameters. For example, a study by Konings et al. [3] found that the 5-Fuorouracil concentrations in the extracellular fluid (ECF) of tumors were lower than the unbound plasma concentrations. Furthermore, especially for central nervous system (CNS) drugs, there exists significant difference between the plasma drug concentration and the CNS drug concentration because of the influence of the blood-brain barrier (BBB). Bostrom et al. [4] showed that the unbound concentrations of oxycodone in brain were higher than those in blood, due to the presence of active influx of oxycodone at the BBB.

Acetaminophen, (N - (4 - Hydroxyphenyl) acetamide), an antipyretic-analgesic drug, is widely used for the treatment of mild pain and fever. The mechanism of acetaminophen hypothermia is not fully understood, but is assumed to be related to inhibit cyclooxygenase in the CNS [5, 6] and the target site locates in the hypothalamus [7]. At recommended therapeutic doses, acetaminophen is safe and effective, however, excessive intake of acetaminophen may cause acute liver failure and even death [8–10]. And acetaminophen overdose is a major cause of liver injury in the USA and Europe [8]. Hence, more accurate and precise pharmacokinetics data of acetaminophen in the CNS (target site) should be obtained. However, so far little research has been done on the pharmacokinetics of acetaminophen at the target site.

Microdialysis, a semi-invasive probe-based sampling technique, is able to measure the unbound drug or endogenous substance concentrations in the ECF of target tissues [11], which is widely used to pharmacokinetics, metabolic as well as tissue distribution studies [1, 12, 13]. Moreover, compared with traditional sampling methods such as tissue biopsy, saliva sampling, skin blister, etc., microdialysis is currently the most appropriate, highly efficient and well-established sampling method [12, 14, 15]. The purpose of this study is to utilize the microdialysis method coupled with high performance liquid chromatography-Ultraviolet detection (HPLC-UV) to analyze the pharmacokinetic process of acetaminophen in the ECF of rats' hypothalamus. And the results would provide suitable references for the clinical development of dosing schedules of acetaminophen. At the same time, it may also establish a new studying method for pharmacokinetics of drugs in the target tissue.

Materials and Methods

Chemicals

Acetaminophen was purchased from Anhui Yongan Pharmaceutical Co. Ltd. Urethane (ethyl carbamate) and propylene glycol were obtained from Shanghai Chemical Reagent Co. Ltd (Shanghai, China) and Hunan Erkang Pharmaceutical Co. Ltd. (Hunan, China), respectively. HPLC-grade methanol andacetic acid were purchased from Tianjin Fu Chen Chemical Reagent Factory (Tianjin, China) and Nanjing Chemical Reagent Co. Ltd. (Nanjing, China), respectively. Artificial cerebrospinal fluid (aCSF) included KCl 3.0 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 1.3 mM, NaCl 140 mM, Na_2HPO_4 2.0 mM and NaH_2PO_4 0.2 mM. Purified water from an AHJZ water purification system was used throughout the experiment. All other chemicals and reagents were of analytical grade.

Animals

Healthy male Sprague-Dawley rats, weighing 250–320g, were purchased from Qing Longshan Animal Breeding Laboratory (Nanjing, China). All animals had free access to food and tap water and were housed at a constant temperature ($25 \pm 2°C$) with a relative humidity ($60 \pm 2\%$) under a 12 h light/dark cycle. All experimental protocols were performed in accordance with the principles of animal use and care approved by the ethnical committee of Wannan Medical College.

Chromatographic System

The chromatographic analysis was carried out on Agilent 1100 LC system (Agilent, USA), coupled to an ultraviolet detection (G1314A). The chromatographic system was used under the following condition: Ultimate XB-C18 column (4.6 × 150 mm, particle size 5μm, USA); mobile phase consisting of phosphate buffer-methanol-glacial acetic acid (90: 10: 0.25, v/v/v) at a flow rate of 1.0 mL/min; injection volume of 20 μL; Column temperature of 25°C and UV detection at 248 nm.

Microdialysis System

The microdialysis system consisted of a microinjection pump (KD Scientific, USA) connected to a 5.0 mL plastic syringe (Hamilton, USA), a MAB 85 fraction collector (Stockholm, Sweden) and a microdialysis probe (EICOM, Japan) which was inserted into the target site.

Preparation of Standard Solutions and Quality Control Samples

The acetaminophen was dissolved in aCSF for obtaining standard final concentrations of 0.25, 0.5, 2.5, 5.0, 10.0, 25.0 μg/mL. Quality control (QC) samples with low (0.5 μg/mL), medium (5.0 μg/mL) and high (25 μg/mL) were also prepared. And then 20 μL of microdialysate samples were injected into the chromatographic system for analyzing.

Selectivity, Linearity and Sensitivity

The selectivity was determined by comparing the chromatograms of blank aCSF sample, blank aCSF spiked with acetaminophen, and microdialysate sample obtained after administration of acetaminophen. To calculate the linearity, the calibration curve with six points in the range of 0.25–25.0 μg/mL was built using peak area of acetaminophen versus acetaminophen concentration. The limit of detection (LOD) was defined as the lowest concentration of analyte and calculated by signal/noise (S/N) ratio equal to 3.

Precision and Relative Recovery

Inter- and intra-day precision were calculated from replicate analysis (n = 5) of QC samples for microdialysate samples, on five consecutive days. The relative recovery was also determined by analyzing the same QC samples in replicate analysis (n = 5). The relative recovery (mean ± SD) was estimated by comparing the measured concentrations to the known concentrations. The relative standard deviation (R.S.D.%) was used to judge the precision.

In vivo Microdialysis Experiment

After rats (n = 5) were anaesthetized by the 20% urethane solution (1.2 g/kg, i.p.) and placed in a stereotaxic apparatus, a microdialysis probe was implanted stereotaxically into the hypothalamus zone (from lambda 4.0 mm posterior, 1.0 mm lateral, 8.0 mm ventral) according to the atlas of George Paxinos & Charles Wastson[16]. The probe was perfused with aCSF solution at a flow rate of 2 μL/min by a microinjection pump. After the probe was allowed to equilibrate for 1 h, the rat was treated with acetaminophen (150 mg/kg, i.p.). Then, the microdialysate samples were collected at 12-min intervals (24 μL) for 6 h and preserved at - 40°Crefrigerator until analysis.

Recovery of Microdialysis Probes

The *in vivo* microdialysis probe recovery was determined by using a retrodialysis method [17]. For *in vivo* recovery, the microdialysis probe was implanted into the hypothalamus zone (above mentioned) of urethane anaesthetized rats. The microdialysis probe was perfused with aCSF solution containing acetaminophen (0.5, 5 and 10 μg/mL, respectively) at a constant flow rate of 2 μL/min by a microinjection pump. Following equilibration 1 h after probe implanted, microdialysates were collected at 12 min intervals for 1 h. And the concentrations of acetaminophen in the perfusate (C_{in}) and dialysate (C_{out}) were determined by the HPLC-UV system. The *in vivo* recovery was calculated by following equation: $R = 1 – (C_{out}/C_{in}) \times 100\%$.

Pharmacokinetics data

The concentrations of acetaminophen in rat microdialysates were determined from the calibration curve. The actual concentration in the ECF of hypothalamus (C_{Hypo}) were calculated from the concentrations in microdialysates (C_{Mdia}) by following equation: $C_{Hypo} = C_{Mdia}/R$. The observed data was used for the calculation of pharmacokinetic parameters by a one-compartment model method using DAS2.0 software. The main pharmacokinetic parameters: elimination of half-life ($t_{1/2}$), peak time (T_{max}), peak concentration (C_{max}), and area under the concentration-time curve (AUC_{last}, AUC_{inf}), etc., were determined. The results are presented as mean ± standard deviation (mean ± SD). All data were analyzed by SPSS 13.0 software.

Results

Selectivity, Linearity and Sensitivity

The acetaminophen's retention time was 7.9 min. The high selectivity was proved by the absence of interfering peak of endogenous compounds around the retention time of acetaminophen (Fig. 1). The calibration curve for acetaminophen in microdialysates (0.25–25.0 µg/mL) was fitted to a linear equation which was A = 57.467 C - 1.9726 (r = 0.9993, n = 5), where A represents the peak area of acetaminophen, and C represents the concentration of acetaminophen. The LOD of acetaminophen for the microdialysate method was 0.25 µg/mL.
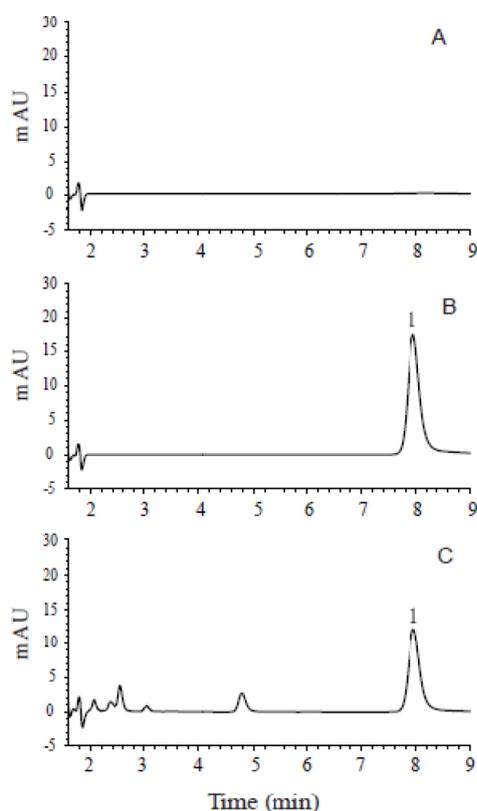
Figure 1: Chromatograms of (A) blank aCSF sample, (B) blank aCSF spiked with acetaminophen (peak 1), and (C) microdialysate sample obtained after administration of acetaminophen (peak 1). aCSF, artificial cerebrospinal fluid.

Precision and Relative Recovery

The inter- and intra-day precision and the relative recovery of the method were shown in Table 1. The inter- and intra-day precision of QC samples were 2.13%, 3.98% ,4.78%, and 2.56%, 3.44%, 6.37%, respectively. The relative recoveries of QC samples were 99.13 ± 2.17%, 99.53 ± 3.49% and 98.20 ± 5.24%, respectively. These results showed that the method had good precision and accuracy.

Recovery of Microdialysis Probe

The probe recovery determined by retrodialysis was showed in Table 2. At the concentrations of 0.5, 5.0 and 10.0 µg/mL (n = 5), the average recovery rate of microdialysis probe was 18.3%.

Table1. The recovery rate and precision of acetaminophen in ECF (mean ± SD, n = 5)

Marked Conc. (µg/mL)	Measured Conc. (µg/mL)	Relative recovery rate (%)	Precision (RSD, %)	
			Inter-day	Intra-day
25.0	24.78 ± 0.54	99.13 ± 2.17	2.13%	2.56%
5.0	4.98 ± 0.17	99.53 ± 3.49	3.98%	3.44%
0.5	0.49 ± 0.03	98.20 ± 5.24	4.78%	6.37%

All means presented are arithmetic.

ECF: Extracellular fluid; **SD**: Standard deviation; **Conc.**: Concentration; **RSD**: Relative standard deviation.

**Table 2.** The recovery rate of microdialysis probe (mean ± SD, n = 5)

C_{in} (µg/mL)	C_{out} (µg/mL)	R (%)
0.50	0.41 ± 0.00	18.27 ± 0.02
5.00	4.08 ± 0.00	18.34 ± 0.02
10.00	8.17 ± 0.00	18.28 ± 0.01

All means presented are arithmetic.

SD, standard deviation; C_{in}, concentration in perfusate; C_{out}, concentration in dialysate.

Pharmacokinetic Process of Acetaminophen

The mean concentration vs. time profile of acetaminophen in the ECF of rats' hypothalamus after administration (150 mg/kg. i.p.) was presented in Figure 2. The concentration-time curve of acetaminophen was fitted to a one-compartment open model, and the main pharmacokinetic parameter estimates were $t_{1/2}$ = (1.95 ± 0.59) h, T_{max} = (1.26 ± 0.22) h, C_{max} = (11.39 ± 2.17) µg/mL, AUC_{last} = (42.93 ± 5.39) µg·h/mL and AUC_{inf} = (58.04 ± 18.39) µg·h/mL, as shown in Table 3.
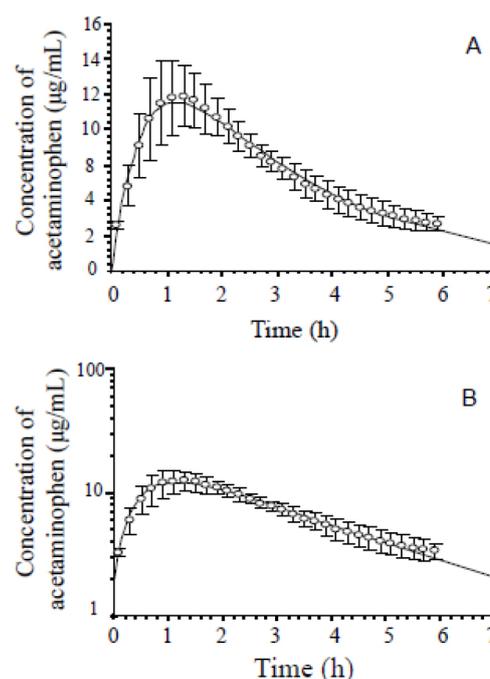
Figure2: Mean C-T curves of acetaminophen in the hypothalamus extracellular fluid of rats after administration of acetaminophen (150 mg/kg, i.p., n = 5). (A) arithmetical C-T curve, (B) logarithmic C-T curve. C-T, concentration-time, error bars indicate standard deviation.

Table 3. Main pharmacokinetic parameters of acetaminophen (150 mg/kg, i.p.) in the ECF of rats (mean ± SD, n = 5)

Parameter	Hypothalamus
$t_{1/2}$ (h)	1.95 ± 0.59
C_{max} (μg/mL)	11.39 ± 2.17
AUC_{last} (μg·h/mL)	42.93 ± 5.39
AUC_{inf} (μg·h/mL)	58.04 ± 18.39
T_{max} (h)	1.26 ± 0.22

All means presented are arithmetic.

ECF, extracellular fluid; **SD** standard deviation; $t_{1/2}$, terminal elimination half-life, C_{max}, maximum concentration; AUC_{last}, area under the concentration–time profile to the last measurable concentration; AUC_{inf}, area under the concentration–time profile from the time of dosing extrapolated to infinity; t_{max}, time to reach maximum concentration.

Discussion

In the present study, we used *in vivo* microdialysis sampling method combined with HPLC-UV to investigate the pharmacokinetics process of acetaminophen in the hypothalamus of rats which is the target site (active-site) of acetaminophen, and obtained some main pharmacokinetic parameters, such as $t_{1/2}$, T_{max}, C_{max}, etc. Compared with data in plasma ($t_{1/2}$, T_{max} and C_{max} were 1.20 ± 0.30 h, 0.58 ± 0.13 h and 97.09 ± 11.08 μg/mL, respectively), the $t_{1/2}$ of acetaminophen in the hypothalamus was prolonged significantly, indicating that acetaminophen eliminated more slowly in the hypothalamus. While the T_{max} in the hypothalamus was 2-fold longer than that in plasma, suggesting the delayed distrib ution of acetaminophen into the hypothalamus, which might be attributed to the presence of the BBB. It is apparent that the C_{max} in plasma was higher than that in the hypothalamus as the acetaminophen concentration in plasma consisted of the free (unbound) and bund concentrations of acetaminophen. All these results suggested that there were significant differences in pharmacokinetic processes between the plasma and target organs/tissues.

In general, the biochemical events and pharmacological effects do not usually take place in the bloodstream, but in target tissues [1, 2]. And active site concentrations of unbound substances are better predictors of drug effects than total plasma or whole tissue concentrations. This is partly due to the presence of active transporters at tissues, but is also due to differences in plasma protein binding and non-specific tissue binding [11]. The active-site concentrations can be defined as the concentrations of unbound, pharmacologically active substances at the site of action. In contrast, the total concentrations of the drug in plasma / organ / tissue also include the protein- or tissue-bound molecules that are pharmacologically inactive [11].

Traditionally, plasma and whole tissue concentrations are used as predictors of effects and side effects, as well as calculating pharmacokinetic parameters because of their ease of sampling, while the concentrations of unbound drug in tissue are more difficult to measure. But just as mentioned above, better predictors of drug effects are the active site concentrations of unbound substances. The results in the present study suggested that there were significant differences between pharmacokinetic parameters based on the plasma drug

concentrations and active-site drug concentrations, implicating that the dosing schedule of acetaminophen in clinic should be designed according to the pharmacokinetic parameters based on the active-site drug concentration for decreasing acetaminophen-causing side effects, i.e. hepatotoxicity. Therefore, it is not accurate and precise to calculate pharmacokinetic parameters using the plasma or whole tissue concentrations for dosing schedule of drugs in clinic.

With the introduction of microdialysis, the first technique with which unbound concentrations could be easily measured *in vivo*. Compared with plasma or tissue sampling, the *in vivo* microdialysis method possesses many advantages. Firstly, *in vivo* microdialysis method can obtain the active site concentrations of unbound drugs [1], which can more accurately predict drug effects and calculate pharmacokinetic parameters. Secondly, the microdialysate samples obtained by *in vivo* microdialysis method can be directly detected by HPLC or HPLC/MS/MS because only low-molecular weight substances can be diffusible through the semi-permeable membrane [2, 18]. Yet, before detecting, the plasma or tissue samples must be dealt with by series of pretreatment, such as protein precipitation, homogenization and centrifugation, etc. These procedures were relatively complex and resulted in unreliable drug concentrations. Finally, *in vivo* microdialysis method can be continuously sampling without loss of the body fluid during the experiment [19], especially small animals such as mice, rats, etc. In brief, *in vivo* microdialysis is a simple, feasible and reliable sampling method, which is of unique advantages in pharmacokinetics study.

For *in vivo* microdialysis method, it is essential to obtain the recovery rate of probe for determining the actual concentration of endogenous drugs or substances, which is determined by several factors such as physicochemical properties of the analyte, semi-permeable membrane materials, diffusion coefficient and temperature [20–23]. The flow rate of microdialysis probes is also a significant factor on the recovery rate of probe increasing with lower flow rate [20]. Generally, the perfusate is usually at a flow rate from 0.1 to 5.0 μL/min. In this study, taking several factors, such as sample volume, sampling time and sensitivity to detect the analyte, into account, we thought that the ideal flow rate was 2 μL/min. Moreover, there are many methods to calibrate the recovery rate of probe, for instance, retrodialysis method [17], external standard method [24], internal standard method [25] and zero-net flux method [26]. In this study, the recovery rate of probe was determined by using the retrodialysis method, the most common calibration method.

This study provided one sample of *in vivo* microdialysis applications in acquiring drug concentration at the target sites for studying pharmacokinetics in rats. In fact, *in vivo* microdialysis has been also widely applied in humans for obtain unbound drug or endogenous substance concentrations in the ECF of target tissues/organs. La Favor et al. [27] successfully utilized microdialysis to measure *in vivo* reactive oxygen species in human skeletal muscle, demonstrating the feasibility of measuring both *in vivo* H_2O_2 and superoxide in the extracellular environment of human skeletal muscle. Simmel et al. [28] provided the proof of principle of long-term subcutaneous microdialysis in humans in which they developed a

special setting to ensure good clinical practice compliance, tolerability, and convenience for participants and personnel. Moreover, Prolonged microdialysis sampling over several days has been used for endogenous compounds and/or drugs in humans in neonatal [29–31] and adult [32–35] diabetic patients, in patients admitted for breast reconstruction with transverse rectus abdominis muscle flaps after mastectomy [36], in patients with ischemic heart disease [37], and in patients for neurochemical monitoring or on the neurosurgical ICU [38]. Hence, unquestionably, with the development of experimental science and technology, *in vivo* microdialysis would be more wide and deep application in basic and clinical pharmacokinetics studies.

Conclusion

Our results reveal that there are significant differences between pharmacokinetic parameters based on the plasma drug concentration and the active-site drug concentration, implicating that the dosing schedule of drugs in clinic should be designed according to the pharmacokinetic parameters based on the active-site drug concentration. And this study provides a simple, feasible and reliable experimental method for studying pharmacokinetics of drug in the target.

Acknowledgement

Conflict of interest

All authors declare no conflict of interest

Ethical approval

All experimental protocols were performed in accordance with the principles of animal use and care approved by the ethnical committee of Wannan Medical College.

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