Estimation of paracetamol in urine to assess the diurnal variation

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Abstract

The aim of the present study was to evaluate the diurnal variation of the pharmacokinetics of paracetamol by estimating the urinary free paracetamol level after single oral administration of paracetamol (500 mg) tablet to 24 healthy male volunteers (students of a Medical College). The volunteers were given paracetamol tablet at 0800, 1400 and 2000 hours in three different days (two weeks apart) and the urine samples of the volunteers were collected at just before and four hours after paracetamol administration. The samples were analyzed for free paracetamol using HPLC. The mean age was 21.1 ± 1.3 years and the body weight was 63.9 ± 10.9 kg. Three peaks were detected in the HPLC and one of them was identified for free paracetamol (RT= 4.7 min). The urine volume was nearly similar in all three times. After administration at 0800 hour, total free paracetamol excretion was significantly more than at 1400 and 2000 hours ($p<0.001$). The present study indicates that the dose reduction of paracetamol is required at morning than the afternoon or evening dose.

Introduction

Diurnal variation is an important phenomenon for human and animal to maintain body homeostasis and for adaptation to the surrounding environment. The diurnal variation occurs around a day and it influences many aspects of the pharmacology. A numbers of drugs, such as antibiotics,[1] antitumor drugs,[2] cardiovascular drugs,[3] immunosuppressive agents,[4] antidepressants[5] and analgesics[6,7] vary significantly in potency and disposition kinetics depending on the time of drug administration.

In earlier studies, diurnal variation of different drugs at different phases of pharmacokinetics has been observed such as drug absorption, distribution, biotransformation and excretion.[8] the safety and efficacy of a drug depend on the dose and concentration of that drug in the body fluid. At different times of the day, the pharmacokinetics of a drug alters, so the safe and effective dose of the drug also varies. The goal of therapy is to achieve the maximum efficacy with least possible adverse effect.

Paracetamol (acetaminophen) is one of the most commonly used drugs in the world but it's diurnal variation was not studied. Though it is considered as one of the safe drugs, it may produce many toxic effects. Total 98,794 cases of adverse drug reactions due to paracetamol have been reported to WHO global ADR monitoring cell, Uppsala, Sweden until September 2017.[9] So, this study was undertaken to know about the diurnal variation of paracetamol and to propose an adjustment of its dose according to this variation for the better efficacy and less toxicity of paracetamol. Chemically, it is n-acetyl-p-aminophenol. It is used widely as an over the counter antipyretic and analgesic drug.[10,11]

Paracetamol is commonly taken orally and absorbed mainly from the small intestine by passive transport.[12] Systemic bioavailability of oral paracetamol is 70-90% and it is dose-dependent.[13] The peak plasma level usually achieves within 30-90 min[14] and in therapeutic dose the plasma concentration varies from 5-20 µg/mL.[15] Generally, paracetamol is prescribed in adults as oral 500 mg tablet for 3-4 times in a day irrespective of body weight and time of administration of a drug. After absorption paracetamol distributes uniformly in the body fluid, the apparent volume of distribution is 0.9 L/kg. Its half-life is 1.6-2.8 hours.[16] Paracetamol is extensively metabolized in the liver by various microsomal and non-microsomal enzymes. Its main metabolic products are sulfuric acid and glucuronic acid conjugates[17] about 30 and 55% respectively. NAPQI (n-acetyl-p-benzoquinone imine) is a toxic intermediate metabolite produced in a small amount which is then detoxified by reduced glutathione and ultimately converted to cysteine and mercapturic acid conjugates. About 2-5% of oral paracetamol remain unchanged[12] which produces its pharmacological action that is the reduction of pain or body temperature. Paracetamol metabolism depends

on the dose and age of the patient.[18] Drug metabolism varies in different races and there is no previous study regarding paracetamol metabolism in the population of Bangladesh. The half-life of paracetamol is also prolonged by chronic liver disease.[19] In men and women, the metabolism of paracetamol, peak plasma level, half-life varies due to the different plasma level of hormones.[20] In case of healthy subjects, 85-95% of the therapeutic dose of paracetamol is excreted in the urine within 24 hours[13] and most of them are within 3-5 hours.[21] At first few hours, the amount of unchanged paracetamol is comparatively more in the urine than the time onwards. Pharmacokinetics of paracetamol follows the first-order kinetics. So, the plasma concentration of paracetamol is directly proportional to its urinary concentration.

The present study has been carried out to compare the urinary excretion of free paracetamol in healthy young men, after 4 hours of ingestion of the same dose of oral paracetamol obtained from the same source at three different times of the day to identify the diurnal changes in the pharmacokinetics of paracetamol in the healthy population. Serum creatinine level was measured for all the healthy volunteers participated in this study to ensure their normal renal function.

Materials and Methods

The study was conducted in Eastern Medical College, Comilla and the Department of Pharmacology, Bangabandhu Sheikh Mujib Medical University from September 2016 to January 2018. A total 24 healthy volunteers were included as study subject by following the inclusion criteria that are: a) Age range of 18-23 years, b) male, c) non-smoker, d) subjects who abstained from paracetamol for 2 weeks, e) subjects who were not taking any enzyme inducing or enzyme inhibiting drug, f) subjects with normal liver and renal function, g) subjects who had no history of hypersensitivity to paracetamol, h) subjects who were interested to participate in this study.

At the commencement of the study, investigator described the whole procedure, objective of this study and the role of volunteers for this study in front of students of Eastern Medical College.

For all volunteers separate data sheets were filled containing demographic data (age, sex), address and personal, family and drug history related to this study.

The volunteers received routine clinical examination including pulse, blood pressure, body-weight, temperature, edema, jaundice, etc and the data were recorded on the data sheet by the investigator. The investigator met the healthy volunteers for intervention and sample collection on three different days at 2 weeks interval. In the first day at 0800 hour, second day at 1400 hour and third day at 2000 hour.

Each volunteer was fasting of at least three hour before administration of paracetamol. The urine was collected in a measuring plastic bottle just before paracetamol administration as baseline sample. The volume was recorded on the data sheet and 10 mL urine was preserved with 0.2 mL chloroform for analysis. Chloroform helps to prevent breakdown of the paracetamol and its conjugates specially the glucuronide.[22] After that, the volunteers were given a single tablet of paracetamol 500 mg at 0800 hour to ingest with a 150 mL of drinking water. Drug intake was ensured by direct supervision. After ingestion of paracetamol, the urine sample was collected 4 hours after drug administration.

Participants were asked to report any adverse effect of the medication given during the study period. In the second day, after two weeks, the same procedure repeated at 1400 hour. Third visit was done at 2000 hour in two weeks after the second visit. Only one brand of paracetamol tablet (Ace 500 mg) made by Square Pharmaceuticals Limited, Bangladesh (purchased from a registered Pharmacy located at Shahbag, Dhaka) was used throughout the study. Samples were carried by maintaining temperature by ice-pack from the Eastern Medical College (84 km from the university) to the Department of Pharmacology, Bangabandhu Sheikh Mujib Medical University. All samples stored at -20°C before analysis.

A Thermo Fisher Scientific DIONEX UltiMate 3000 series (Waltham, USA) was used for the analysis and quantitation of paracetamol in the urine. The Dionex Chromeleon software management system controls the whole chromatographic system. Paracetamol was separated on a reversed-phase chromatographic column TCC-3000SD and TCC-3000RS, column-18 fitted with a pre-column (Thermo Fisher Scientific Inc. USA) using combined isocratic and linear gradient elution. Acetonitrile and isopropyl alcohol were obtained from the Daejung Chemicals & Metals Co. Ltd. (Korea). Methanol and chloroform obtained from the Merck (Germany). Distilled deionized ultrapure HPLC grade water was prepared in the laboratory by water deionzation system and filtered through Pall Life Science filter paper 0.2 µm. All liquids used were HPLC grade. Standard paracetamol was kindly gifted from the Square Pharmaceuticals Ltd. (Bangladesh). Chromatographic separation was achieved on a reversed phase C18 column (19 × 42 × 51 cm). Column and injection temperatures were both maintained at 25° C. The system was operated at a flow rate of 0.5 mL/min. Injection volume was 10 µL. The UV detector was set at 254 nm. The mobile phase was

prepared fresh daily with acetonitrile and distilled water in a ratio 10:90. Liquids used for the mobile phase were measured with measuring cylinder and kept in a glass beaker. After that, it was filtered through Pall Life Science filter paper 0.2 µm and then sonicated through Sonorex Super 10 P for 3 min.

The urine samples were kept at room temperature after taken out from refrigerator until they became in liquid. From each container 200 µL of the urine sample was taken with a micropipette to a test tube and 200 µL acetonitrile added to it and mixed properly with the vortex mixer. Then the sample was centrifuged for 10 min at 3,500 rpm. After centrifugation 200 µL supernatant was collected in a separate test tube and 200 µL of mobile phase solution was added to it. Then this was mixed properly with vortex mixer and taken to small glass vial for injecting 10 µL into the HPLC system. Identification of peaks in samples was executed by comparing with those peaks which are derived from aqueous standard solution. One milligram of working standard of paracetamol was measured with an electronic digital balance and dissolved in 1 mL of HPLC grade distilled water with the help of vortex mixer. It was than diluted with mobile phase to prepare stock solution 100 µg/mL. All solutions were protected from light and were stored at 4°C.

The standard calibration was done before estimation of the urine samples. The limit of detection calculated for the estimation of paracetamol was 0.3 µg/mL and the limit of quantification was 0.8 µg/mL. After measuring the urinary paracetamol from each sample, baseline value was deducted and then the concentration in each mL multiplied by the total volume of urine after paracetamol intake to estimate the total urinary excretion of paracetamol of each individual. The mean of the differences of urinary concentration paracetamol of all 24 subjects was calculated in three different time of the day for statistical purpose.

After taking aseptic precaution, 4 mL of blood was collected in a vacuum tube from each healthy volunteer. The tube was kept standing for one hour and then centrifuged for 10 min at 3,500 rpm to prepare the clear serum. After that, serum was collected in separate Eppendorf tube and carried with icepack and stored at –20°C temperature. This serum was then used for the estimation of serum creatinine concentration by alkaline picrate method by using spectrophotometer. The confounders in the present study were: a) Influence of food, b) formulation of drug, c) drug interaction, d) posture of subject, and e) physical exercise. To minimize these factors, healthy volunteers were kept fast for at least three hours before and half-an hour after the administration of paracetamol tablet. They were requested to eat and drink the same type food (meal supplied in hostel dinning) in the days of sample collection. They were supplied 500 mg paracetamol tablet prepared by same manufacturer (square pharmaceutical limited, Bangladesh) with same batch No. (6G00749). Any probable drug that can interact with the study drug were avoided by the study subjects as far as possible. They were advised to maintain sedentary posture and avoid any physical exercise in the day of study. Regular counseling was done to improve the adherence to the treatment and thus to limit the confounding variables as much as possible. Education and proper counseling, direct supervision of drug intake, subject's interview over mobile phone were done for better adherence to the study.

Statistical analysis

Statistical analysis of the values was done by paired student's t-test with quick statistic calculator.

Results

Among the 44 enrolled non-smoker native Bangladeshi participants, 20 healthy volunteers (5 participants have withdrawn from the study for their absence; 15 received paracetamol medication within the study period) were dropped out. The mean (± SD) age of the participants was 21.1 ± 1.3 years and the body weight was 63.9 ± 10.9 kg. Among the 24 healthy volunteers, 5 received other drugs such as omeprazole, albendazole, fluconazole, vitamin B complex etc. during or within two weeks of study period. Two participants complained of mild pain in abdomen and malaise after administration of paracetamol. None of the participant was alcoholics and their mean serum creatinine concentration was 0.8 ± 0.2 mg/dL.

The interval of urine sample collection after administration of paracetamol tablet at 0800 hour (morning) was 240.9 ± 10.4 min whereas the interval of urine sample collection after 1400 hour (afternoon) and 2000 hour (evening) administration was 235.5 ± 8.0 and 237.3 ± 9.2 min (Table I). There was no significant differences in the time of urine sample collection after administration at three differ-ent times. The volume of urine after paracetamol administration at the morning, afternoon and evening were 202.1 ± 107.1, 185.4 ± 103.7 and 196.9 ± 118.3 mL respectively which were nearly similar. But there were significant inter-individual variations in urinary volume at three different times. There were three definite peaks detected in the HPLC during the urine sample run for 10 min. Peak 1 and 3 remained unidentified due to lack of standard chemical. Peak 2 which was identified as the peak for free paracetamol. It was more detected after at the morning sample (10.7%) than that afternoon sample (4.4%) and evening sample (8.4%). From the area of peak 2 in the urine sample and standard free paracetamol, urinary concentration of

Table I

Findings of the urine samples of participants (n=24)

Parameters	Time of paracetamol administration (Hour)			p value
	0800	1400	2000	
Urine collection time (min) after administration	240.9 ± 10.4	235.5 ± 8.0	237.3 ± 9.2	a=0.070 b=0.272 c=0.525
Urinary volume after paracetamol administration (mL)	202.1 ± 107.1	185.4 ± 103.7	196.9 ± 118.3	a=0.502 b=0.757 c=0.679
Retention time of peak 2 (min)	4.59 ± 0.86	4.55 ± 0.89	4.52 ± 0.88	a=0.482 b=0.024 c=0.546
Area of peak for paracetamol in HPLC	7377 (10.7%)	3535 (4.4%)	5133 (8.4%)	a<0.001 b=0.051 c=0.068
Urinary paracetamol concentration (µg/mL)	52.0 ± 37.0	23.8 ± 21.2	31.6 ± 25.8	a<0.001 b=0.007 c=0.059
Total urinary free paracetamol in 4 hours after oral paracetamol tablet administration (mg)	9.0 ± 6.1	4.1 ± 3.1	5.0 ± 3.3	a<0.001 b<0.001 c=0.091

Data were presented as mean ± SD; Here, a= 0800 vs 1400 hour; b= 0800 vs 2000 hour; c= 1400 vs 2000 hour

free paracetamol was measured. It was calculated as 52.0 ± 37.0, 23.8 ± 21.2 and 31.6 ± 25.8 µg/mL in the morning, afternoon and evening sample respectively (Table I). The concentration of paracetamol in urine was highest after administration at morning and more standard deviation signifies there was wide interindividual variation. Total free paracetamol measured in urine of morning was 9.0 ± 6.1 mg and it was 4.1 ± 3.1 and 5.0 ± 3.3 mg at afternoon and evening sample respectively. The concentration of free paracetamol at morning was significantly more than that measured at afternoon and evening (p= 0.0002 and 0.0008).

Discussion

In this study, a significant diurnal variation of free paracetamol in the urine sample of the healthy volunteers was found. All the volunteers were male and they were within a same age group. The mean area of the peak for free paracetamol was more prominent after the oral paracetamol tablet administration at morning and it was 10.7% of three detected peaks. After administration at afternoon and evening, it was 4.4 and 8.4% respectively. The total peak area was highest at afternoon and lowest at evening administration. All the urine samples were collected near about four hours after paracetamol administration and there was no significant difference in the volume of urine collected at the three different times. But the concentration of urinary free paracetamol and the total urinary excretion of free paracetamol varied significantly in between the morning, afternoon and evening administration. Difference between the 8 AM and 2 PM administration was found most significant (p=0.0002), whereas the difference between the afternoon and evening sample found non-significant (p=0.0911). The excretory functions of the kidneys of volunteers were normal, as all of their serum creatinine levels were within normal range.

In a study regarding factors affecting paracetamol absorption, it was observed that the mean urinary excretion of paracetamol was 36% less when the oral paracetamol tablet (1 g) administered to four volunteers at 11 PM than that at 8.30 AM.[23] In the same study, it was also found that the decreased rapidity of paracetamol absorption when it was administered just after the food intake. In the present study, urinary concentration and the total amount of free paracetamol was more (p=0.0002 and 0.0008) after administration of paracetamol at morning than that afternoon and evening samples. Paracetamol tablet was administered at all three times to the volunteers who were kept fast at least three hours before and half-an-hour after ingestion of paracetamol. So, the increased urinary free paracetamol at morning administration may be due to lack of metabolism rather due to increased absorption. The highest total peak area but less peak area for free paracetamol in urine at afternoon administration indicates more absorption and metabolism of paracetamol at that time. The lowest total peak area indicates lack of absorption of

paracetamol after administration at evening and this result was similar to an earlier study.[23]

The mean plasma half-life of paracetamol was found 15% more after oral administration of paracetamol at 6 AM than at 2 PM in a study.[24] Inter-individual variation is another important thing which was observed in that study where the half-life of paracetamol varied from 34% decrease to 1.8% increase among eight study subjects administered oral paracetamol at 6 AM and 2 PM. Plasma half-life was not measured in this study but the interindividual variation in urinary free paracetamol was found as indicated by the high value of standard deviations.

Another study was done in South Africa on six healthy non-smoker male volunteers with the administration of 1 g oral paracetamol at 8 AM, 2 and 8 PM at the one-week interval found no significant difference in the pharmacokinetic parameters that were $t_{1/2}$, T_{max}, C_{max}, and AUC.[25] This study was carried out under rigidly controlled and standardized condition. The pharmacokinetic parameter of our study was different from this study but found significant diurnal variation. This difference may be due to variation in race, the number of volunteers or lack of standardization.

A significant diurnal variation was found in urinary excretion of paracetamol glucuronide in a study regarding the diurnal variation of paracetamol absorption and metabolism. Urinary paracetamol glucuronide was highest after oral paracetamol administration at 8 AM and almost doubled in amount than administration at 8 PM.[26] Urinary excretion of free paracetamol and other metabolites of paracetamol did not display any significant diurnal variation in that study, whereas in present study diurnal variation in urinary free paracetamol was observed. A significant variation found in the mean peak salivary paracetamol concentration and time to reach the peak concentration in that study. This difference may be due to the genetic difference in the study population as the pharmacokinetics in Asian people is different from Caucasian population.[27, 28]

Serum creatinine is one of the most important markers of renal function which may influence on the excretion of paracetamol through urine.[29] In the present study, serum creatinine levels of all the volunteers were within normal range. So, it did not seem to cause any influential change in urinary paracetamol.

All the studies before this study were done with very small study population and the used dose was 1.0-1.5 g paracetamol. The studies were done in different population and the results were not uniform. In this study, a relatively large number of the volunteer was recruited and usually practicing therapeutic dose for Bangladeshi population (500 mg tablet) was used for the study.

Conclusion

There is a significant diurnal variation of urinary paracetamol excretion after the oral administration of paracetamol. Low paracetamol metabolism and high free paracetamol excretion in urine after oral administration at morning than at afternoon or evening indicating less requirement of oral paracetamol at the morning than other two times of the day.

Ethical Issue

The protocol was approved by the Institutional Review Board of Bangabandhu Sheikh Mujib Medical University on January 9, 2017 (BSMMU/2017/324) and registered on the website clinicalTrials.gov (NCT 03122561). An approval was taken from the Principal, Eastern Medical College to allow the medical students as healthy volunteers to participate in this study. Written informed consent was taken from the volunteers.

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