CIRCADIAN RHYTHMS OF PARACETAMOL METABOLISM IN HEALTHY SUBJECTS; A PRELIMINARY REPORT

J.M. Ngong and R.H. Waring*

School of Biochemistry, University of Birmingham Edgbaston, Birmingham B15 2TT, U.K.

SUMMARY

Paracetamol was used as a "probe" drug to study the circadian rhythms of metabolite ratios in man. Paracetamol was orally administered to six volunteers at different times of day, 0-8 h and 8-24 h urine samples being measured for sulphate and glucuronide formation. Results showed a wide interindividual variation in paracetamol metabolite excretion among the six subjects. However, when a 500 mg dose was administered, free paracetamol excretion was minimal when the dose was given at 12.00 h and maximal when given at 20.00 h for the 0-8 h collection period. Sulphate excretion rose slightly at night and decreased gradually during the day. Glucuronide excretion was greatest with drug administration at 16.00 h and least if paracetamol was ingested at 08.00 h. The 8-24 h profiles were roughly similar.

At a higher dose (1500 mg), free paracetamol excretion showed a minimum from dosing at 20.00 h and a maximum from dosing at 24.00 h in both 0-8 h and 8-24 h collections, while the sulphate conjugate peaked for doses at 20.00 h and 8.00 h with collections at 0-8 h and 8-24 h respectively. The glucuronide conjugate was maximal for paracetamol administration at 16.00 h for both 0-8 h and 8-24 h collections.

There appears to be a 12 hour phase variation in excretion; this may result from circadian rhythms in absorption and enzyme activities. These parameters may also affect metabolism at higher dose levels, so that the hepatotoxicity of paracetamol could vary with the time of dose.

KEY WORDS: paracetamol, metabolism, human, circadian

* Author for correspondence

INTRODUCTION

Circadian rhythms in metabolism and pharmacokinetics have been reported for many drugs /1-4/ and have been linked with fluctuations in enzyme levels /2,5/ or protein binding /6/. Paracetamol (acetaminophen, N-acetylamino-4-phenol) at low dosage is largely excreted as the glucuronide and sulphate conjugates, with small amounts of the parent compound /7,8/, and various workers have used it as a "probe" drug to measure factors affecting metabolite ratios. The formation of the sulphate conjugate is believed to be determined by the supply of inorganic sulphate, as the concentration of this anion is rate-limiting /9,10/. However, serum sulphate levels have been reported to show a circadian rhythm in man, being lowest in the morning (8-11 a.m.) and reaching a maximum in the early evening (~ 7.00 p.m.) /11/. The present study was undertaken to determine whether urinary excretion of paracetamol sulphate varied in accordance with these parameters.

MATERIALS AND METHODS

Subjects

Six subjects (4 females mean age 37, range 26-47, 2 males ages 30 and 38, all subjects of normal weight [55-70 kg]) volunteered to participate in the study. They were in good health and had no evidence of renal or hepatic dysfunction. All were non-smokers and none had been taking any medication at the time of study or for three weeks previously. Alcohol, paracetamol, aspirin and medications of any type were not permitted throughout the study periods. Ethical Committee approval was obtained for the study.

Study protocol

All volunteers took a 500 mg (Study 1) or 3 x 500 mg tablet of paracetamol (Study 2) (Queen Elizabeth Hospital, Birmingham Pharmacy Dept.) with at least 100 ml of water on six occasions (five occasions for study 2), each one week apart. Times of dose were 0.00 h, 04.00 h, 08.00 h, 12.00 h, 16.00 h and 20.00 h, so that paracetamol was taken at 4-hourly intervals around the 24-hour day. Urine samples were collected and pooled for a) the first eight hours after paracetamol ingestion and b) the following 16 hours, to give 0-8 and 8-

24 h urine collections. The total volumes were measured and 15 ml aliquots were stored at -20°C pending analysis. Volunteers were not synchronised for food intake, although all kept to a standard diet on the days of the experiment, nor for sleep/wake times, although again a standard protocol was adhered to as much as possible with paracetamol ingested on an empty stomach and a sleep period of ~ 10.30 p.m. - 7.00 a.m. There was no correlation of urinary volume with metabolism.

Paracetamol and conjugate assay

Paracetamol and its glucuronide and sulphate metabolites were measured by standard methods /12-14/; other metabolites were not significant (<2% of dose) at the doses used. An aliquot (1 ml) of urine was transferred into a test tube containing 200 µl of the internal standard acetanilide (Sigma Chemical Company, U.K.), and 250 µl of methanol (HPLC grade, Fisons, U.K.). The mixture was vortex mixed for 1 minute and 50 µl of sample injected onto the HPLC system.

Hydrolysis of sulphate metabolite

An aliquot (1 ml) of sample was transferred into a test tube containing 1.2 ml of acetate buffer (pH 5) and a small amount of sulfatase (~ 10 mg) (Sigma, U.K.) at the end of a spatula. The brand of sulfatase used (S-9626) contained some glucuronidase activity which was prevented by adding a small amount (5 mg) of the glucuronidase inhibitor D-saccharic acid-1,4-lactone (Sigma, U.K.). Samples were then incubated in a water bath at 37°C for 16-18 hours. After hydrolysis, samples were treated as above. Glucuronide metabolites were similarly estimated by incubation with glucuronidase (bovine β-glucuronidase type B1, 1000 units) and the paracetamol so released was estimated as above. The glucuronide and sulphate metabolites are not commercially available so release of free paracetamol (which parallels the decrease of the conjugate peak) is the standard accepted method.

HPLC conditions

The HPLC system for the analysis of paracetamol consisted of a Rheodyne 7125 injector (Catoli, CA, USA), a Waters pump, model 6000A (Milford, MA, USA), a Techsphere 5 ODS column (HPLC

Technology Ltd., Macclesfield, U.K.) (250 x 4 mm I.D., 5 μ particle size), a Pye Unicam variable wavelength LC3 UV detector, and a chart recorder (Labdata, U.K.).

The mobile phase consisted of 1% (v/v) acetic acid, methanol, ethyl acetate (90:15:01 v/v). The flow rate was set at 1.6 ml/min and the absorbance of the eluate was monitored at 254 nm.

Using these conditions, baseline resolution was obtained for paracetamol and acetanilide, with retention times at approximately 6 minutes and 17 minutes respectively. Calibration curves for paracetamol in urine were constructed for the ranges 5-100 μg/ml. The coefficient of variation over these concentration ranges was 7.5% at the lowest levels, and 4.6% (5-15 μg/ml) at higher values, with a correlation coefficient of 92.2%.

Analysis of data

The concentrations of free (non-hydrolysed) paracetamol, paracetamol sulphate and paracetamol glucuronide were determined in all samples. The total sulphate and glucuronide levels were obtained by subtracting the amount of free paracetamol from the increase observed after enzymic hydrolysis.

The percentage dose of free drug with its sulphate and glucuronide conjugates was then calculated for each sample, the recovery being determined by adding all the results together.

Plots of percentage of dosage versus time of drug administration were then constructed, the values being given for the initial time of drug administration, so that figures plotted for any given time represent those found for the succeeding 0-8 h or 8-24 h period. Statistical analysis was performed using the non-parametric Wilcoxon Rank Sum method.

RESULTS

The results after a 500 g dose of paracetamol (Study 1) are shown in Figures 1-4, the values being plotted with respect to time of drug administration. Although there is wide variation between volunteers, it can be seen that in the 0-8 h period after administration, the excretion of free paracetamol was minimal when the drug was given at 12.00 h and maximal when given at 20.00 h (Fig. 1). Excretion of the sulphate conjugate was relatively invariant, with values slowly decreasing

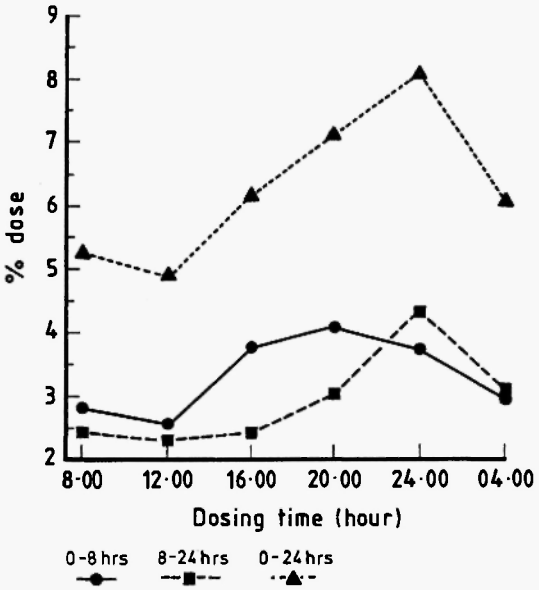

Fig. 1: Urinary excretion of free paracetamol after a 500 mg dose administered at different times of day.

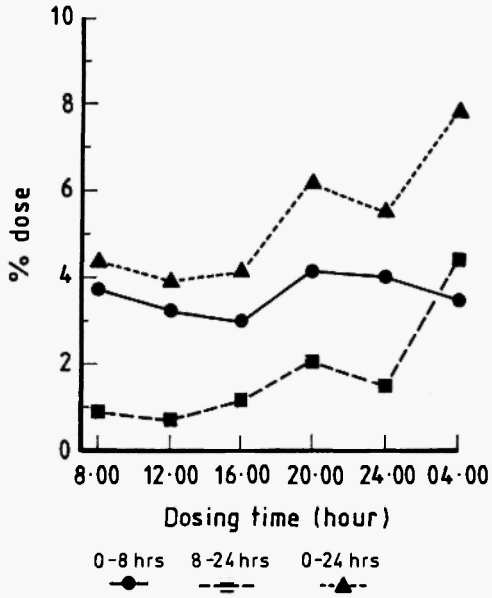

Fig. 2: Urinary excretion of paracetamol sulphate after a 500 mg dose administered at different times of day

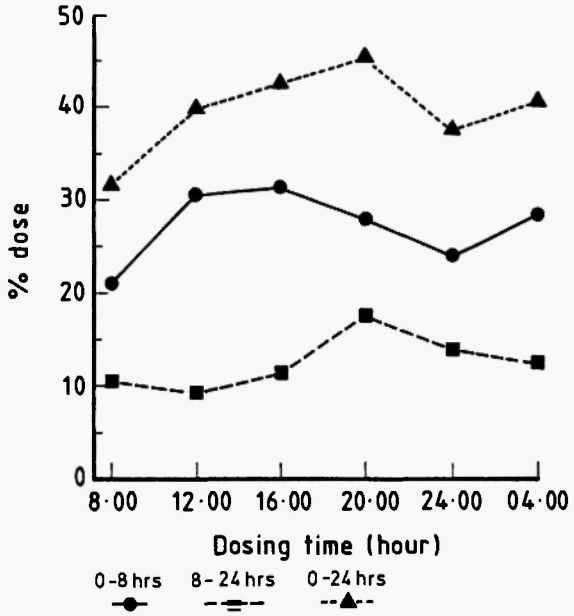

Fig. 3: Urinary excretion of paracetamol glucuronide after a 500 mg dose administered at different times of day.

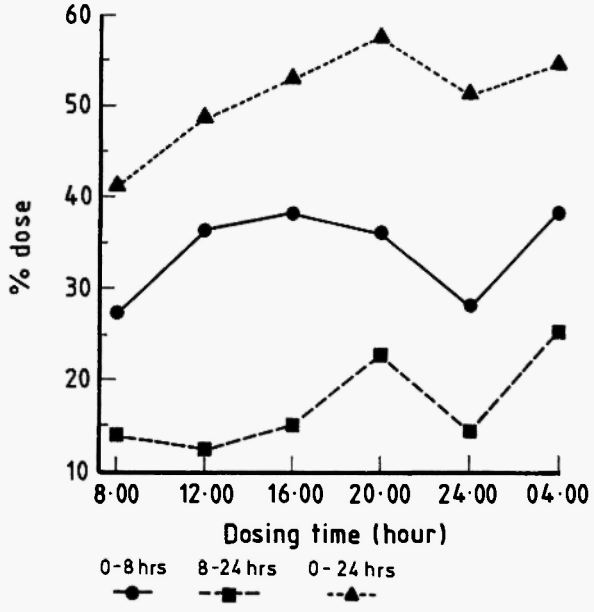

Fig. 4: Urinary excretion of total paracetamol and its metabolites after a 500 mg dose administered at different times of day.

throughout the day and rising slightly at night (Fig. 2). Glucuronide excretion was minimal after administration at 08.00 h and maximal after administration at 20.00 h (Fig. 3). However, when the 8-24 h period is analysed, excretion of free paracetamol and both conjugates also showed circadian variation, the sulphate conjugate peaking after administration at 4.00 h and 20.00 h and the glucuronide metabolism after administration at 20.00 h.

Total recoveries over the 0-24 h period also showed some variation, a minimum occurring after administration at 08.00 h (Fig. 4). Full values are given in Table 1.

Results from volunteers after a 1500 mg dose (Study 2) are shown in Figures 5-8. In the 0-8 h period minimum values of free paracetamol were excreted after administration at 20.00 h and maximum values after administration at 24.00 h (Fig. 5). Paracetamol sulphate excretion showed a peak after administration at 20.00 h which was significantly higher than results for other dosing times (P=0.02) (Fig. 6). Excretion of the glucuronide conjugate showed a peak after administration at 16.00 h (Fig. 7).

Analysis of the 8-24 h urine collection period in the higher dose study showed free paracetamol excretion to peak after administration at 24.00 h and to have minimum levels after administration at 20.00 h. Highest levels of the sulphate metabolite were found after administration at 8.00 h (P=0.03 with respect to other sampling times), and the glucuronide after administration at 16.00 h. Glucuronide excretion showed a significant (P=0.05) increase after administration at 16.00 h when results were pooled to give 8-24 h excretion.

Total recoveries at this higher dose level showed little fluctuation during the 0-8 h period and peaked after administration at 16.00 h for the 8-24 h period (Fig. 8). Full values are given in Table 2.

DISCUSSION

The topic of circadian changes of drug disposition in man has been reviewed /14/ and several studies have investigated temporal variation in paracetamol metabolism in man. Shively and Vessel /4/ demonstrated a small prolongation of paracetamol half-life in healthy individuals given a single dose of the drug at 06.00 h as opposed to 14.00 h, although there was no variation in urinary recovery of the glucuronide conjugate. However, other workers /15/ found no

TABLE 1
Percentage of dose excreted as free paracetamol, sulphate, glucuronide and total recovery after a 500 mg dose. Values represent mean ± standard deviations with the ranges shown in brackets.

	Time of Dosing	Free Paracetamol	Sulphate	Glucuronide	Total Recovery
0-8HRS	8.00hr	2.82+1.96 (0.97-4.90)	3.46+2.5 (1.17-6.13)	20.98+11.02 (9.73-35.62)	27.3+17.75 (9.18-49.16)
	12.00	2.56+2.81 (1.11-5.91)	3.2+3.7 (0.22-7.35)	30.52+21.55 (8.78-53.62)	36.3+26.7 (11.14-65.31)
	16.00	3.76+2.81 (1.22-7.1)	2.97+2.13 (0.63-5.84)	31.23+21.3 (10.11-56.72)	38.0+24.5 (12.01-62.91)
	20.00	4.08+2.8 (0.98-7.2)	4.13+2.4 (1.58-6.82)	27.7+25.7 (8.51-62.73)	35.9+29.1 (14.14-70.11)
	24.00	3.73+2.4 (1.26-6.3)	4.01+4.65 (0.35-9.13)	23.8+15.2 (16.71-46.32)	27.97+15.8 (15.65-49.82)
	4.00	2.96+2.06 (0.87-5.2)	3.46+3.27 (0.21-7.37)	28.2+8.76 (19.82-43.41)	38.05+13.35 (16.39-42.37)
8-24HRS	8.00	2.43+1.6 (0.73-4.9)	0.89+0.6 (0.37-1.73)	10.5+2.88 (8.17-14.37)	13.84+3.35 (11.73-21.62)
	12.00	2.3+2.85 (0.21-6.10)	0.7+0.83 (0.11-1.82)	9.24+3.7 (5.71-13.63)	12.33+7.27 (9.16-22.54)
	16.00	2.42+2.4 (0.37-5.11)	1.14+1.3 (0.17-2.56)	11.31+7.1 (5.01-19.62)	14.86+7.87 (6.31-23.72)
	20.00	3.03+2.6 (0.42-0.71)	2.04+4.4 (0.07-8.11)	17.48+8.95 (8.28-27.11)	22.55+13.15 (91.62-38.42)
	24.00	4.33+4.04 (0.62-9.93)	1.48+1.8 (0.11-3.73)	13.75+7.33 (6.71-22.61)	14.13+2.03 (10.17-17.62)
	4.00	3.11-2.1 (0.86-6.34)	4.4+6.27 (0.01-12.18)	12.3+6.67 (4.32-17.16)	48.6+25.3 (20.91-69.73)
0-24HRS	8.00	5.26+2.9 (2.07-9.31)	4.36+5.92 (0.03-10.62)	31.6+13.03 (10.41-50.17)	41.13+2.03 (35.72-44.18)
	12.00	4.88+3.84 (1.76-10.32)	3.91+3.85 (0.07-8.42)	39.8+21.15 (16.41-62.13)	48.6+25.3 (22.63-77.14)
	16.00	6.18+4.64 (1.34-10.52)	4.11+2.45 (1.81-7.36)	42.5+26.3 (21.31-71.11)	52.8+28.9 (19.62-80.17)
	20.00	7.11+4.4 (2.72-12.58)	6.17+4.83 (2.2-11.62)	45.2+25.6 (18.37-63.41)	57.3+29.2 (30.16-82.42)
	24.00	8.07+6.23 (3.11-14.97)	5.5+6.2 (0.08-14.73)	37.4+20.9 (16.92-58.10)	51.0+29.3 (23.56-79.96)
	4.00	6.07+3.81 (2.06-11.11)	7.82+6.45 (1.92-15.81)	40.45+8.01 (26.17-49.12)	54.33+12.6 (40.08-71.32)

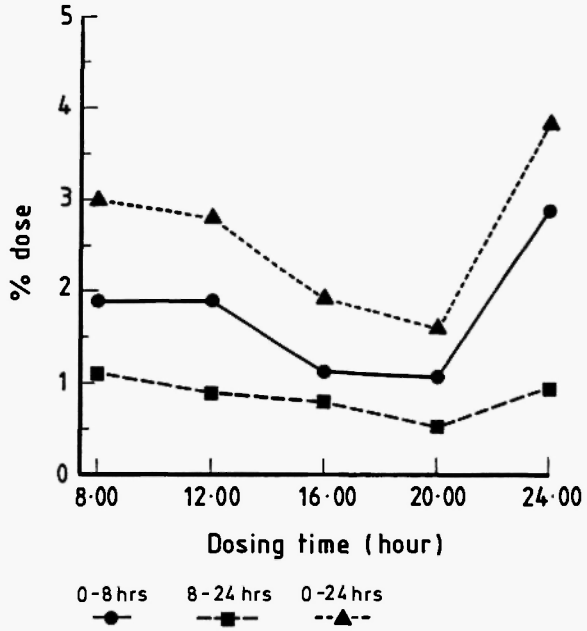

Fig. 5: Urinary excretion of free paracetamol after a 1500 mg dose administered at different times of day.

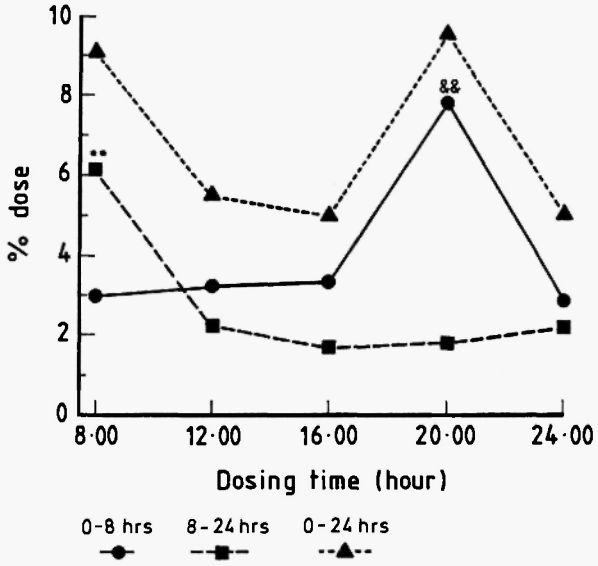

Fig. 6: Urinary excretion of paracetamol sulphate after a 1500 mg dose administered at different times of day. ** p=0.03; && p = 0.02.

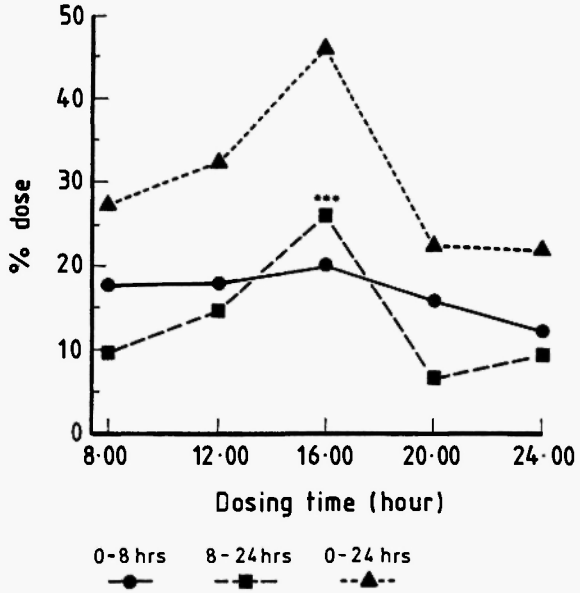

Fig. 7: Urinary excretion of paracetamol glucuronide after a 1500 mg dose administered at different times of day. *** p=0.05.

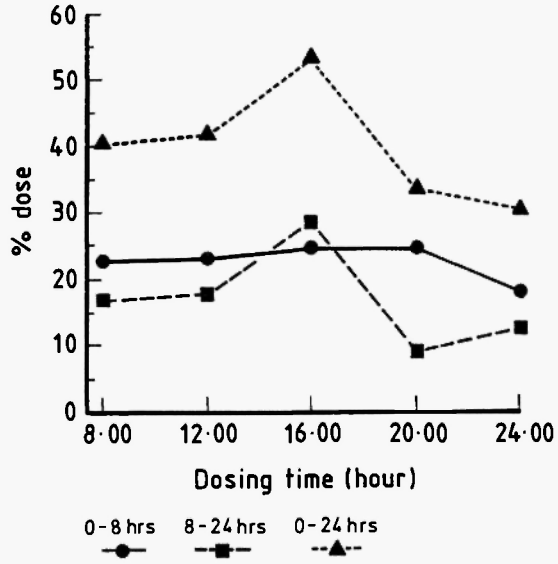

Fig. 8: Urinary excretion of total paracetamol and its metabolites after a 1500 mg dose administered at different times of day.

TABLE 2

Percentage of dose excreted as free paracetamol and its metabolites and total recovery after a 1500 mg dose. Results represent the mean ± standard deviation with the ranges shown in brackets.

	Time of Dosing	Free Paracetamol	Sulphate	Glucuronide	Total Recovery
0-8HRS	8.00hr	1.89±0.86 (0.92-3.0)	2.98±1.32 (1.83-4.55)	17.64±5.81 (14.4-29.3)	22.6±6.52 (14.4-29.3)
	12.00	1.9+0.61 (1.5-2.6)	3.2±0.67 (2.62-5.3)	17.85±3.85 (13.6-22.7)	22.94±4.85 (17.72-29)
	16.00	1.3±0.921 (0.3-2.11)	3.3+0.85 (2.3-4.9)	20.05+6.73 (12.6-29.6)	24.56+9.36 (16.77-35.4)
	20.00	1.07±0.54 (0.6-1.64)	7.76+1.9 (5.6-9.5)	15.78+6.73 (10.05-23.2)	24.61+6.0 (18.3-32.7)
	24.00	2.9+0.4 (1.68-3.8)	2.83+0.78 (1.8-4.2)	12.2+1.43 (10.98-14.1)	17.93+1.05 (22.15-24.2)
8-24HRS	8.00	1.1+0.813 (0.5-2.3)	6.12±3.04 (3.5-10.5)	9.63±3.04 (6.3-13.54)	16.85+3.96 (13.7-22.0)
	12.00	0.89±0.44 (0.6-1.4)	2.2+0.48 (1.84-2.9)	14.58+8.6 (7.5-24.13)	17.67±8.07 (10.1-28.43)
	16.00	0.8±0.63 (0.3-1.5)	1.64+0.66 (0.96-2.5)	26+39.3 (5.5-85)	28.44±1.3 (8.0-10.3)
	20.00	0.53+0.2 (0.34-0.73)	1.75+0.58 (1.38-2.6)	6.6+1.43 (5.4-8.13)	8.9+3.75 (7.24-15.7)
	24.00	0.95±0.66 (0.46-1.8)	2.16+0.54 (1.56-2.8)	9.29±8.32 (7.8-10.2)	12.4±1.02 (29.4-51.3)
0-24HRS	8.00	2.99±1.05 (1.8-4.0)	9.10±3.92 (4.85-14.1)	27.3±8.32 (17.63-34.5)	40.27+10.14 (29.4-51.3)
	12.00	2.8±0.46 (2.3-3.2)	5.47+1.04 (4.64-6.87)	32.43+11.67 (16.2-37.7)	41.69±5.08 (34.4-46.15)
	16.00	1.93+0.71 (1.3-2.7)	4.96±1.5 (4.2-6.7)	46.05+9.24 (19-37.5)	53.05+10.55 (24.85-45.7)
	20.00	1.6+0.46 (1.08-1.98)	9.52±1.96 (6.7-11.2)	22.38±3.26 (18.15-24.32)	33.51±5.42 (29.4-41.14)
	24.00	3.85±0.97 (1.27-5.6)	5.02+0.72 (4.1-5.8)	21.9±1.68 (20.9-24.33)	30.33±2.17 (37.0-42.11)

differences in serum half-life for a 1 g dose of paracetamol given at 08.00 h, 14.00 h and 20.00 h. Kamali *et al.* /16/, looking at 1.5 g doses in man, found more glucuronide in the 0-4 h urine when the drug was given at 08.00 h and the lowest amount from dosing at 20.00 h. They attributed their findings to a diurnal variation in absorption, suggesting that posture may play a part. Other investigators /17/ found that there was no circadian variation in absorption for a number of water-soluble drugs, including paracetamol. It has been shown /10/ that the oral systemic availability of paracetamol is ~80%, independent of the dose level, while the gastrointestinal tract is not an important site for its metabolism. Our results for the 0-24 h excretion of the sulphate conjugate after a 500 mg dose appear to show that although the 0-8 h elimination of this metabolite is fairly constant, later levels may correlate with the fluctuations reported by Hoffman *et al.* /11/, who found a slight elevation of serum sulphate in the evening with lowest levels in the morning. This is consistent with the known circadian variation of cysteine dioxygenase, the major source of inorganic sulphate /18/. Peak values for the 8-24 h period in our study occurred with night-time administration and reduced values were found for daytime dosing. However, as in general the 0-8 h profiles for metabolite excretion are not very dissimilar from the 8-24 h figures, the variations seen throughout the 24 h day may simply be a function of a circadian rhythm in kidney metabolism. This has been discussed by Reinberg and Smolensky /4/; variations in urinary pH may be involved as well as fluctuations in re-absorption or excretion in the renal tubule and in urinary flow-rate.

The data reported here show that there is a small but consistent variation of metabolism of paracetamol over a 24-hour period, and suggest that, particularly at higher doses, paracetamol hepatotoxicity may be more severe if the drug is taken at a particular time of day. Liver damage is believed to be due to reactive intermediates which deplete hepatic glutathione (GSH) and so cause tissue injury. On the basis of these results, minimal metabolism (and hence maximal GSH depletion) would occur if the paracetamol was taken around midnight; doses taken around 16.00 h would seem least likely to lead to hepatic damage. A study with a larger number of subjects is needed to verify these findings.

REFERENCES

1. Belenger PM. Chronobiological variation in the hepatic elimination of drugs and toxic chemical agents. Ann Rev Chronopharmacol 1987; 4: 1-46.
2. Radzialowski FM, Bousquet WF. Daily rhythmic variation in hepatic drug metabolising enzymes in the rat and mouse. J Pharmacol Exp Ther 1968; 163: 229-238.
3. Shively CA, Vessell ES. Temporal variations in acetaminophen and phenacetin half life in man. Clin Pharmacol Ther 1975; 18: 413-424.
4. Reinberg A, Smolensky MH. Circadian changes of drug disposition in man. Clin Pharmacokinet 1982; 7: 401-420.
5. Harris BE, Song RL, He YJ, Scong SJ, Diasio RB. Circadian rhythm of rat dihydropyridine dehydrogenase. Biochem Pharmacol 1988; 37: 4759-4762.
6. Hecquet B, Meynadie J, Bonnelerre J, Adenis L. Circadian rhythm in cisplatin binding on plasma protein. In: Reinberg A, Smolensky M, Labreque G, eds, Ann Rev Chronopharmacol. Oxford: Pergamon Press, 1984; 123-138.
7. Prescott LF. Kinetics and metabolism of paracetamol and phenacetin. Br J Clin Pharmacol 1980; 10: 291-298.
8. Steventon GB, Heafield MTE, Waring RH, Williams AC, Sturman S, Green M. Metabolism of low dose paracetamol in patients with chronic neurological disease. Xenobiotica, 1990; 20: 117-122.
9. Levy G. Sulphate conjugation in drug metabolism. Fed Proc 1986; 45: 2235-2240.
10. Clements, JA, Critchley JA, Prescott LF. The role of sulphate conjugation in the metabolism and disposition of oral and intravenous paracetamol in man. Br J Clin Pharmacol 1984; 18: 481-485.
11. Hoffmann DA, Wallace SM, Verbeeck RK. Circadian rhythm of serum sulfate levels in man and acetaminophen pharmacokinetics. Eur J Clin Pharmacol 1990; 39: 143-148.
12. Howie D, Adrianssens PI, Prescott LF. Paracetamol metabolism following overdosage: application of HPLC. J Pharm Pharmacol 1977; 29: 235-237.
13. Nakamura J, Baba S, Nakamura T, Sasaki H, Shibasaki J. A method for the preparation of calibration curves for acetaminophen glucuronide and acetaminophen sulphate in rabbit urine without authentic compounds in high performance liquid chromatography. J Pharmacobio-Dynam 1987; 10: 673-677.
14. Lemmer B. Chronopharmacology: a new perspective in pharmacology. Pol J Pharmacol Pharm 1987; 39: 435-449.
15. Malan J, Moncrieff J, Bosch E. Chronopharmacokinetics of paracetamol in normal subjects. Br J Clin Pharmacol 1985; 19: 843-845.
16. Kamali F, Fry GR, Bell GD. Temporal variation in paracetamol absorption in man. Xenobiotica 1987; 17: 635-642.
17. Reinberg A, Smolensky MR. Circadian changes of drug disposition in man. Clin Pharmacokinet 1982; 7: 401-420.

18. Mitchell SC, Waring RH. S-Oxygenases IV: human pharmacogenetics. In: Damani LA, ed, Sulfur-containing Drugs and Related Organic Compounds, Vol. 2B. Chichester: Ellis Horwood, 1989; 101-119.