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The Effect of Timing of a Single Injection on the Toxicity of Methotrexate in the Rat

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Summary. *The possibility of a circadian rhythm in the toxicity of methotrexate was investigated in rats after a single intravenous bolus. Indices of haematological, renal and hepatic toxicity were studied, as were the pharmacokinetics of the drug. All the parameters showed a circadian variation, with maximum toxicity occurring after dosage at 06.00 h and minimum toxicity after dosage at midnight. Administration at the other two time points, 12.00 h and 18.00 h, gave intermediate results.*

Introduction

Folic acid analogues have been used in the treatment of neoplastic disease since 1948 [8] and methotrexate is now one of the most widely used drugs in the treatment of leukaemia, severe psoriasis, and some solid tumours. The level of the dose and the regularity and duration of treatment have been severely limited by the toxic side-effects of the drug, which range from the unpleasant to the fatal. The two most commonly occurring unwanted effects are leukopenia and stomatitis, the former being the limiting factor in the further treatment of 81% of one series of patients [13]. Other common toxic effects include kidney damage resulting in elevated serum creatinine and blood urea nitrogen levels [7] and hepatotoxicity characterised by raised serum transaminase levels [2]. In more recent years the use of high-dose methotrexate therapy has been made possible by the addition of leucovorin rescue regimens in which the toxic side-effects of methotrexate are minimised by treatment with the reduced folate derivative, calcium leucovorine [18]. However, the interpatient variation in both susceptibility to methotrexate and plasma clearance of the drug means that toxic effects are still a considerable problem [25].

Substantial evidence exists of a circadian rhythm in drug response especially in rodents, particularly in the effects of pentobarbital sodium [26], amphetamines [24], and diazepam [19]. Similar variations have been shown in the toxicity of some cytotoxic drugs, notably cytosine arabinoside [12, 14], adriamycin [17, 21], and cisplatin [16]. The toxicity and cure rate of cytotoxic drug combinations when used to treat L1210 murine leukaemia have been shown to exhibit a circadian rhythmicity [4, 22]. There are some indications that the clinical response to adriamycin and cisplatin in patients with advanced ovarian carcinoma can be improved if the drugs are administered at specific circadian stages [16]. Survival rates of patients with a variety of solid tumours treated by chronochemotherapy with methotrexate or 5-fluorouracil, followed by vinblastine and cyclophosphamide, were similarly improved [10].

In view of these findings we decided to investigate the possibility of a circadian variation in the toxicity and pharmacokinetics of methotrexate in the rat as part of a longer study into the influence of timing on drug metabolism, pharmacodynamics, and pharmacokinetics.

Materials and Methods

All experiments were performed on male Norwegian hooded rats weighing 250–300 g and maintained on a lighting schedule of 06.30–18.30 h light, 18.30–06.30 h dark.

Pharmacokinetic data were obtained after a methotrexate dose level of 2 mg/kg body weight. Groups of six rats were anaesthetised with Sagatal (0.1 ml/100 g body weight, ip) and methotrexate (Lederle) dissolved in 0.9% saline made slightly alkaline (pH 7.5–8.0) with $NaHCO_3$ was injected through an exposed femoral vein. Blood samples (0.2 ml) were removed from the tail vein at 0, 5, 10, 20, 30, 40, 50 and 60 min after administration of the drug and allowed to clot. The serum was stored at −20° C until assayed for methotrexate concentration by radioimmunoassay [1]. Pharmacokinetic data were derived by conventional methods [27].

Toxicity data were obtained after a single iv injection of methotrexate at a dose level of 200 mg/kg body weight. The drug was given as described above and blood samples were obtained from the tail vein at 12-h intervals up to 96 h after injection and then at 24-h intervals up to 240 h. Total white cell count was determined by cytometry, serum creatinine level by the Jaffé method [23], serum urea by Berthelot's reaction after cleavage with urease [9], and serum glutamic oxaloacetic transaminase (SGOT) by a colorimetric method [20].

Both sets of experiments were performed on four occasions, with the methotrexate injected at 06.00 h, 12.00 h, 18.00 h or 24.00 h.

The statistical significance of any differences found was tested using Student's *t*-test for paired observations, comparing each time point with the basal value for that parameter.

Results

Pharmacokinetics

The mean plasma elimination half-life ($t_{1/2}$) of methotrexate between 10 and 60 min after injection varied from 13.5 ± 0.2 min when the drug was administered at 24.00 h to 22.0 ± 1.7 min when it was given at 06.00 h; the areas under the serum

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Table 1. Toxicity and pharmacokinetic data[a] in rats given methotrexate iv at four different times of the day

		Time of administration			
		6.00 h	12.00 h	18.00 h	24.00 h
(a) Toxicity (dose level 200 mg/kg body weight)					
Deaths		3/6	0/6	0/6	0/6
Total white cell count (10^9/l)	Pre-experimental	7.2 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1
	Lowest	2.2 ± 0.2****	3.5 ± 0.2****	3.6 ± 0.1****	5.1 ± 0.1****
	10 days post dose	2.8 ± 0.2****+	6.8 ± 0.2*	4.9 ± 0.2****	7.1 ± 0.1
Urea (mmol/l)	Pre-experimental	6.8 ± 0.5	7.4 ± 0.2	6.5 ± 0.3	7.4 ± 0.2
	Highest	16.6 ± 0.7	8.9 ± 0.5*	10.9 ± 0.6****	7.7 ± 0.3
	10 days post dose	15.5 ± 0.9****+	6.6 ± 0.3	7.7 ± 0.6	7.1 ± 0.3
Serum creatinine (mmol/l)	Pre-experimental	113 ± 4	121 ± 4	111 ± 1	120 ± 1
	Highest	183 ± 8****	159 ± 3****	155 ± 2**	130 ± 3***
	10 days post dose	156 ± 6**+	118 ± 2	111 ± 4	121 ± 3
SGOT (SF units)	Pre-experimental	93 ± 5	96 ± 1	63 ± 5	93 ± 6
	Highest	249 ± 25****	138 ± 10***	156 ± 7****	107 ± 4
	10 days post dose	226 ± 31**+	138 ± 10***	130 ± 6****	107 ± 4
(b) Pharmacokinetics (dose level 2 mg/kg body weight)					
	$t^1/_2$ min	22.0 ± 1.7	16.0 ± 0.6	18.0 ± 1.2	13.5 ± 0.2
	AUC (μg/ml·h^{-1})	6.5 ± 0.4	5.5 ± 0.3	5.6 ± 0.6	3.3 ± 0.2
	Peak plasma level (μg/ml)	18.7 ± 2.7	12.3 ± 1.1	12.3 ± 1.1	7.0 ± 0.6

[a] Values given are means ± SEM for six animals except where marked. +, mean ± SEM for three animals. * $P > 0.05$; ** $P > 0.01$; *** $P > 0.005$; **** $P > 0.001$

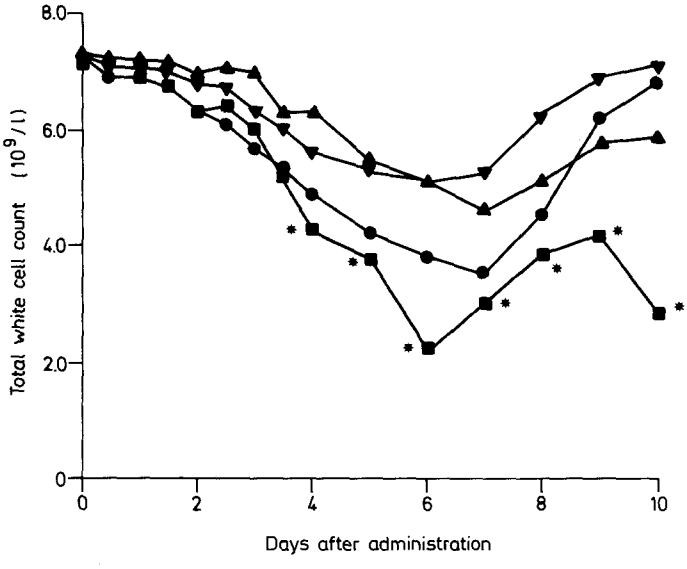

Fig. 1. Effect of a single iv bolus of methotrexate (200 mg/kg) given at different circadian points on white cell counts in the rat. Each *point* represents the mean of values recorded in six rats, except where marked *; such *points* represent means of values recorded in three rats. Time of administration: (■) 06.00 h; (●) 12.00 h; (▲) 18.00 h; (▼) 24.00 h

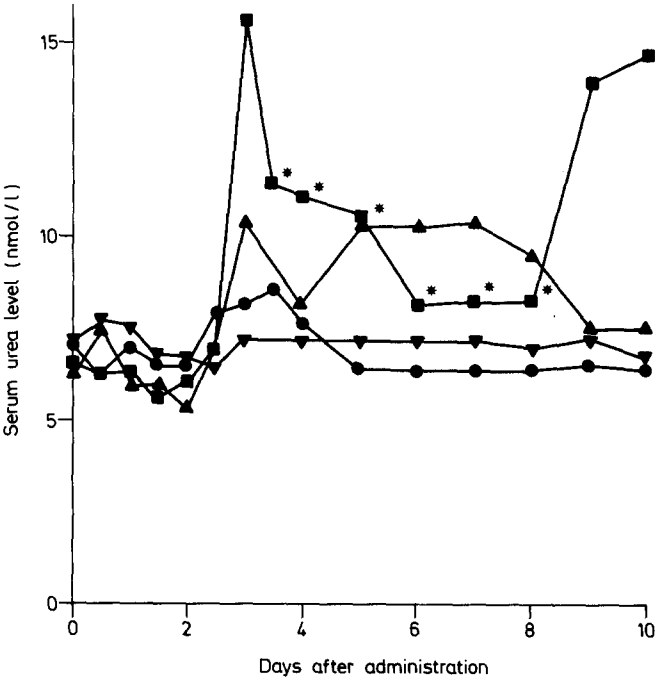

Fig. 2. Effect of a single IV bolus of methotrexate (200 mg/kg) at different circadian points on serum urea levels in the rat. Each *point* represents the mean of values recorded in six rats, except where marked *; such *points* represent means of values recorded in three rats. Time of administration: (■) 06.00 h; (●) 12.00 h; (▲) 18.00 h; (▼) 24.00 h

concentration time curve showed a parallel increase from 3.25 ± 0.24 μg/ml · h^{-1} to 6.50 ± 0.43 μg/ml · h^{-1} at the same two time points. The pharmacokinetic data derived are shown in Table 1.

Toxicity

The most obvious result of the investigation into the toxicity of methotrexate was that 3/6 of the rats dosed at 06.00 h died between 3 and 4 days later, and the remainder of that group were in poor condition at the termination of the experiment, whilst none of the other animals showed any undue distress.

All four groups of rats showed signs of myelosuppression, but whilst the group dosed at 24.00 h showed an average maximum suppression of 27% in the total white cell count and

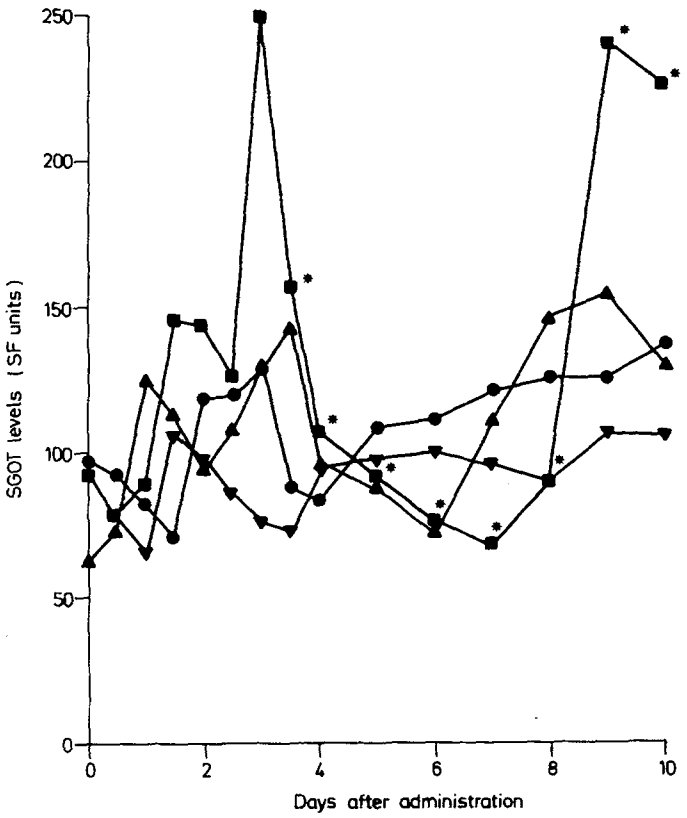

Fig. 3. Effect of a single iv bolus of methotrexate (200 mg/kg) given at different circadian points on serum glutamic oxaloacetic transaminase levels in the rat. Each *point* represents the mean of values recorded in six rats, except where marked *; such *points* represent means of values recorded in three rats. Time of administration: (■) 06.00 h; (●) 12.00 h; (▲) 18.00 h; (▼) 24.00 h

had recovered by 10 days after administration of the methotrexate, the group dosed at 06.00 h showed a mean maximum reduction of 68% and at the termination of the experiment the number of white cells was still 59% lower than before the drug was given. The other two groups showed intermediate results (Fig. 1).

Serum creatinine and blood urea concentrations showed similar patterns. The animals dosed at 06.00 h showed considerably higher levels of both compounds than any other group and those dosed at 24.00 h showed little or no rise in either. Blood urea levels are shown in Fig. 2.

The rise in SGOT was greatest in rats given methotrexate at 06.00 h and least in those dosed at 24.00 h. The results are shown graphically in Fig. 3.

Discussion

Our results indicate that there is a well-marked time-related variation in the severity of all the toxic manifestations of methotrexate that were monitored. The maximum effect in each case occurred after administration of an iv bolus of drug at 06.00 h; and the minimum effect occurred when the drug was given at midnight. The severity of the toxic reaction bore a direct relationship to the peak plasma methotrexate level, the AUC and the $t_{1/2}$.

It was not possible to determine the reasons for the variability in drug handling from these studies.

Methotrexate is metabolised to only a minor extent in rodents and is excreted largely unchanged in roughly equal amounts in the urine and in the bile [15]. The portion excreted in the bile is extensively metabolised by bacteria in the caecum and the products either excreted in the faeces or absorbed into the enterohepatic circulation [28]. Variations in plasma clearance rate could therefore be caused by variations in either renal filtration rate or biliary output. The former is known to be subject to a diurnal variation in rats, with a maximum glomerular filtration rate at midnight corresponding to the point of most rapid plasma clearance [5, 6]. Renal clearance rates could also be influenced by fluid intake or urinary pH, either of which may vary in a diurnal manner in the rat. Variation in the extent to which methotrexate is bound to plasma proteins could also influence its clearance rate, since only free methotrexate is available for renal or hepatic excretion [3].

The major route for methotrexate excretion in man is through the kidney: most of the drug is excreted unchanged after its rapid iv injection, though some is converted to 7-hydroxymethotrexate. It remains to be seen whether these differences in the excretory pattern eliminate the time-determined variation in pharmacokinetics observed in the rat and whether they affect frequency and severity of toxic side-effects: the fact that the toxicity of methotrexate depends on the duration of its persistence in the blood, rather than on its peak level, suggests that $t_{1/2}$ and toxicity are closely related in man [11].

Since the reduced toxicity observed at times other than 06.00 h appear to be consequent upon increased rates of plasma clearance, presumably the tumour cells of an affected organism would also be exposed to lower levels of methotrexate. This might well impair the desired cytotoxic effect of the drug. If, as now seems likely, the toxicity can be reduced by administering methotrexate according to a circadian schedule, the efficacy of such a regimen would have to be investigated before the clinical relevance of our findings can be assessed.

An incidental side observation to emerge from this work is that the LD_{50} of methotrexate varied widely with the time of its administration. If this is true of other drugs it casts further doubts on the utility and scientific validity of LD_{50} tests, unless these tests are performed at various stated circadian stages.

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