Circadian variation in biochemical assessments used to monitor rheumatoid arthritis

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SUMMARY Circadian variation has been demonstrated in several clinical parameters used to assess disease activity in rheumatoid arthritis, but circadian variation in modern laboratory assessments has not been studied in depth. We therefore made 2-hourly measurements of plasma viscosity, C-reactive protein, total serum sulphhydryl, and serum histidine on samples obtained over a 24-hour period from 6 patients with classical or definite rheumatoid arthritis. Hourly control samples were also taken from 6 normal volunteers, 3 of whom starved from 2200 h the previous night and 3 of whom ate normally. There was no significant variation in any of these laboratory measurements between 0900 and 1800 h either in patients or controls. These findings enable us to eliminate circadian variation as a source of error when using these laboratory tests in clinical trials of slow-acting anti-rheumatoid drugs.

There have been several reports showing pronounced circadian variation in the clinical assessments used to assess disease activity in rheumatoid arthritis (RA).¹⁻³ This variation in disease activity has implications for our understanding of disease pathogenesis and for the way in which clinical trials are performed. For example, Kowanko *et al.*⁴ have shown that the efficacy of flurbiprofen differs according to the time of day and to the doses administered. When a drug is being assessed over an extended period, as in the case of a slow acting antirheumatoid drug (SAARD), it is important that clinical changes noted at each visit are due to the effect of the drug and not to other factors such as the time of day or observer error.⁵

In addition to clinical measurements, clinical trials of SAARDs usually employ laboratory tests in order to provide a more objective assessment of disease activity and to help in the detection of drug-related side effects. Some of these laboratory variables are also subject to circadian variation. One group of investigators has recently demonstrated a diurnal variation in the erythrocyte sedimentation rate (ESR) in patients with RA which was related to the

ingestion of food.⁶ No statistically significant change in C-reactive protein (CRP) was noted in any of the patients during the day whether they were eating a standard hospital diet or fasting. Harkness *et al.*³ have found significant circadian variations in C1q binding, peripheral neutrophil count, and plasma cortisol concentrations.

We have previously shown that plasma viscosity (PV), CRP, serum histidine, and total serum sulphhydryl are useful measurements to make during the assessment of SAARDs.⁷ In addition gamma glutamyl transpeptidase (GGTP) is raised in 20-30% of patients with RA⁸ but is often measured to help detect hepatic toxicity during treatment of RA with immunosuppressive agents such as azathioprine.

We have investigated whether any of these laboratory variables show a tendency to vary with the time of day during a period of 24 hours in 6 RA patients and over 12 hours in 6 normal volunteers.

Materials and methods

PATIENTS

Six patients (4 female, 2 male, mean age 60.3 yr, SD 13.0 yr) were included in the study. All had classical or definite RA (American Rheumatism Association criteria), and the group had a mean erythrocyte sedimentation rate of 53 (SD 27) mm/h. Each patient

Accepted for publication 22 July 1983.

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Table 1 RA patient details

Case	Age (yr)	Sex	Rheumatoid factor (RAHA)	Westergren ESR (mm h ⁻¹)	Drug Therapy			
					0600	1200	1800	2200
1	65	F	+	67	Bumetanide, cimetidine, digoxin paracetamol	Ferrous sulphate	Cimetidine	Indomethacin, danthron, paracetamol
2	73	M	—	73	Indoprofen Moduretic*	Paracetamol Indoprofen	Paracetamol Indoprofen	Indoprofen
3	63	F	+	90	Aloxiprin	Aloxiprin	Aloxiprin	Aloxiprin indomethacin
4	64	M	—	32	Bendrofluazide, acebutolol, aloxiprin	Aloxiprin	Aloxiprin	Indomethacin aloxiprin
5	35	F	+	35	Salsalate indomethacin, D-penicillamine	Salsalate	Indomethacin, D-penicillamine	Salsalate
6	62	F	+	21	Carbamazepine, aloxiprin	Carbamazepine (1400) Aloxiprin	Aloxiprin	Carbamazepine, naproxen

*Hydrochlorothiazide and amiloride HCl.

had breakfast at 0800 h, lunch at 1200 h, and tea at 1730 h. Meals were not standardised but were similar for each patient. Drug therapy (Table 1) was administered at 0600, 1200, 1800, and 2200 h, and regular medication was continued unchanged. Blood samples were taken at 2-hourly intervals over a period of 24 hours.

Six normal volunteers (4 female, 2 male, mean age 25.7 yr, SD 5.54 yr) agreed to have hourly blood samples taken over a 12-hour time period starting at 0900 h. They were divided into 2 groups. Group 1 comprised 3 volunteers who agreed to starve from 2200 h the previous night until 1900 h on the study day. This was done to determine the effect of food on the laboratory variables during a normal working day. Group 2 comprised 3 subjects who had their normal food intake during the study day.

Table 2 Reproducibility and sample storage conditions for the biochemical assays

Assay	Reproducibility within batch (%)	Sample storage	
		Temperature °C	Expiry time
CRP	10.0	-20	6 Months
Plasma viscosity	0.5	Room temp.	3 Days
GGTP	6.2	-20	6 Months
Histidine	4.0	-20 within 4 hr of venipuncture	6 months
Sulphydryl	2.7	-20	1 Week

BIOCHEMICAL METHODS

A 15-ml venous blood sample was taken from each patient at the appropriate sample time. Five ml with EDTA was used for measuring PV and GGTP, and 10 ml, clotted, for the remaining tests. After centrifugation the resulting plasma and serum samples were stored according to the conditions shown in Table 2.

Plasma viscosity was measured with a Coulter-Harkness viscometer.⁹ All measurements were made at 25°C with reference to a standard solution of 3.6% saline. Plasma GGTP concentrations were recorded with a Pye Unicam SP/1800 ultraviolet spectrophotometer.¹⁰ CRP was measured by single radial immunodiffusion¹¹ using laboratory prepared plates containing CRP antibody (Hoechst Pharmaceuticals), each plate having 36 wells. Total serum sulphydryl levels were measured spectrophotometrically by an interchange reaction with 5, 5' dithiobis (2-nitrobenzoic acid).¹²

Serum histidine concentration was assayed by a modification of the method of Gerber¹³ employing a Perkin-Elmer LS3 spectrofluorimeter. The method is based on the fluorescence emitted by the complex formed between *o*-phthalaldehyde and histidine in an alkaline solution. The samples were analysed in duplicate at 1½-minute intervals to exclude the artificial increases in fluorescence after termination of the reaction with hydrochloric acid.

Quality control samples with predetermined mean and standard deviation, from at least 10 replicates, were employed during the measurement of serum

Fig. 1 Comparison of the circadian variation in total serum sulphhydryl between group 1 and group 2 volunteers.
◆ Group 1. ● Group 2.

histidine, GGTP, and CRP. If a quality control sample was found to be outside ± 2 standard deviations of the mean, the batch was discarded and repeated.

STATISTICAL METHODS

One-way analysis of variance was used to compare the changes in each biochemical variable over 24 hours in RA patients and over 12 hours in normal volunteers. Student's paired *t* test was also used to compare biochemical parameters between subjects who ate normally and subjects who starved.

Results

No statistically significant variation was seen in laboratory variables among the RA patients or the normal volunteers.

Plasma viscosity in the RA patients showed a downward trend from 0900 to 0300 h with a slight

rise from 1.79 to 1.82 cP at 1900 h, but these changes were not statistically significant. There was little variation in the normal volunteers, but group 1 had significantly higher values for PV ($p < 0.02$) than group 2 (Student's *t* test).

Total serum sulphhydryl rose slightly in RA patients from 1100 to 2100 h and fell until 0300 h, when it started to rise again. Among the normal subjects group 1 had significantly lower ($p < 0.001$) total serum sulphhydryl than group 2. Results ranged between 450–490 $\mu\text{mol/l}$ in group 1 and 475–535 $\mu\text{mol/l}$ in group 2, showing little circadian variation (Fig. 1).

The GGTP levels (Tables 3 and 4) did not vary much over the time period for the RA patients or normal volunteers.

In the RA patients serum histidine had lower values between 2300 h and 0700 h than at other times of the day (Fig. 2). The mean levels of serum histidine

Fig. 2 Comparison of the circadian variation in serum histidine in 6 RA patients and 6 normal volunteers. $\triangle$ Mean of 4 normal volunteers. $\blacktriangle$ Mean of 6 normal volunteers. $\bullet$ Mean of 6 rheumatoid patients. (SI conversion: serum histidine $\text{mg}/100 \text{ ml} \times 10 = \text{mg/l}$).

Table 3 Laboratory measurements (mean $\pm$ SEM): normal volunteers

Laboratory variable	n	Time (h)															
		0900	1000	1100	1200	1300	1400	1500	1600	1700	1800	1900	2000	2100	2200	2300	
Plasma viscosity cP	4	1.58 (0.06)	1.56 (0.05)	1.59 (0.08)	1.57 (0.04)	1.56 (0.05)	1.55 (0.05)	1.55 (0.05)	1.58 (0.05)	1.61 (0.07)	1.56 (0.04)	1.57 (0.05)	1.56 (0.07)	1.57 (0.07)	ND	ND	
GGTP, units/l	4	44 (26)	42 (25)	47 (28)	47 (27)	47 (28)	41 (27)	44 (27)	47 (26)	42 (25)	40 (23)	38 (22)	42 (26)	44 (27)	ND	ND	
Serum histidine, mg/dl	6	1.36 (0.06)	1.32 (0.08)	1.31 (0.08)	1.34 (0.06)	1.43 (0.07)	1.40 (0.04)	1.47 (0.07)	1.47 (0.05)	1.39 (0.05)	1.36 (0.04)	1.40 (0.02)	1.41 (0.05)	1.54 (0.04)	1.52* (0.08)	1.65* (0.06)	
Serum Sulphydryl μ mol/l	6	515 (13)	495 (14)	490 (17)	500 (22)	480 (22)	480 (16)	490 (7)	485 (22)	505 (18)	495 (22)	495 (17)	490 (18)	480 (17)	490* (17)	500* (12)	

* Mean of 4 normal volunteers. ND = no data.

Table 4 *Laboratory measurements (mean $\pm$ SEM): patients with RA*

Laboratory variable	n	Time (h)											
		0900	1100	1300	1500	1700	1900	2100	2300	0100	0300	0500	0700
Plasma viscosity cP	6	1.88 (0.06)	1.81 (0.07)	1.81 (0.06)	1.80 (0.07)	1.79 (0.08)	1.82 (0.03)	1.80 (0.07)	1.74 (0.05)	1.75 (0.06)	1.70 (0.06)	1.75 (0.05)	1.81 (0.08)
GGTP, units/l	4	36 (11)	28 (8)	33 (10)	34 (10)	36 (12)	27 (7)	31 (8)	40 (6)	32 (7)	32 (9)	32 (9)	40 (11)
Serum histidine, mg/dl	6	1.21 (0.06)	1.29 (0.09)	1.21 (0.09)	1.23 (0.07)	1.24 (0.09)	1.19 (0.04)	1.34 (0.07)	1.17 (0.07)	1.14 (0.03)	1.17 (0.08)	1.14 (0.07)	1.15 (0.04)
Serum Sulphhydryl μ mol/l	6	340 (35)	305 (35)	335 (30)	315 (30)	340 (30)	370 (30)	380 (40)	335 (35)	325 (30)	300 (30)	305 (30)	325 (30)
CRP mg/dl	6	2.60 (1.13)	2.39 (1.13)	2.49 (1.11)	2.64 (1.15)	2.84 (1.18)	2.74 (1.15)	2.82 (1.20)	2.69 (1.16)	2.85 (1.22)	2.63 (1.14)	2.61 (1.07)	2.05 (0.87)

were a little low for the normal volunteers, as 2 of the serum histidine profiles included had very low levels of 1.19 and 1.21 mg/dl at 0900 h, which then rose to 1.71 and 1.53 mg/dl in the afternoon (SI conversion: mg/dl $\times$ 10 = mg/l).

It was not considered worthwhile to measure CRP in normal volunteers, as concentrations would be expected to be below the detection limit of the assay (<0.05 mg/dl). CRP levels in the RA patients (Table 4) were slightly higher at night than during the day but they did not vary significantly.

Discussion

Feigin *et al.*¹⁴ have noted that the total concentration of amino acids in human blood varies rhythmically during the course of a 24-hour day, but the precise role of numerous individual hormones and other regulatory mechanisms in maintaining normal amino acid periodicity remains undefined. Hussein *et al.*¹⁵ concluded that the pattern of daily fluctuations in plasma free amino acid levels is significantly affected by the dietary conditions under which the measurements are made. Evidence for the effects of previous histidine intakes on plasma histidine has been presented by Kopple and Swendseid.¹⁶ In their study a group of normal persons were fed an isonitrogenous diet (40 g protein) followed by a low histidine diet (60 mg/day) for 35 ± 2 days and a histidine replete diet (1200 mg/day) for 31 ± 5 days. Plasma levels fell rapidly and markedly with the low histidine diet. However, with the histidine replete diet plasma levels rose slowly and often took several weeks to plateau.

Histidine is present in carnosine (β -alanyl-L-histidine) in a variety of animals and serves as a reservoir during histidine deficiency.¹⁷⁻¹⁹ Other adaptive responses to low intakes of histidine have been reported. They include decreased oxidation and degradation¹⁹⁻²¹ and reduced synthesis of haemoglobin,^{16,22} and some histidine may be synthesised in mammals, including adult humans.^{23,24} It is unique among essential amino acids in that with dietary deprivation nitrogen balance usually does not become negative, and adverse symptoms appear only after 15-30 days of a histidine deficient diet. Even then the balance is usually not markedly negative.²⁵ Therefore it is not surprising that the histidine profiles of groups 1 and 2 were not statistically different. The serum histidine levels of the RA patients did not seem to vary in the same way as the normal volunteers, which may be a consequence of the underlying disease or of drug treatment. Nevertheless it does mean that a blood sample can be taken at any time

during the normal working day, 0900-1700 h, for a reliable measurement of serum histidine to be made, particularly in the context of a clinical trial. Total serum sulphhydryl, which is to some degree a reflection of protein concentration, might be expected to be lower in group 1. However, it does not vary much throughout the day.

Another variable which did not show much variation during the working day is plasma viscosity. It was slightly raised in group 1, and this may be due to mobilisation of fats. It is a measurement which is gaining recognition as a superior alternative to the ESR in the assessment of disease activity in RA.^{26,27} Mallya *et al.* have found significant diurnal variation in ESR relating to ingestion of food which may be due to the effects of feeding on the dielectric coefficient of plasma.⁶ These results have important implications in the use of ESR both in routine practice and in monitoring the effects of therapy. Their data strengthen the case for using PV rather than ESR to monitor the acute-phase response in RA.

The standard errors remain fairly constant in all the groups, so it is unlikely that individual patients are showing circadian variation. These errors are large for the RA patients because the variables were originally selected to distinguish different levels of disease activity in patients. In using the F ratio for testing for significance, the large variation within the RA group is counteracted by the number of sampling times.

Several clinical variables used to monitor RA show circadian variation. For example, there is marked variation in grip strength totalling 70-230 mmHg,¹ while Harkness *et al.*³ found disease activity as measured by joint pain, stiffness, articular index, and grip strength to be maximum between 0200 and 0400 h and minimum in the early afternoon.

The biochemical parameters did vary slightly with the time of day, and a larger sample size may show a significant variation in some cases. However, blood samples could be taken any time between 0900 and 1700 h for a fair representation of disease activity.

The variation found in the clinical parameters has important implications in the dosage, timing, and administration of drugs to achieve maximum efficacy. In contrast our data suggest that the use of biochemical assessments in clinical trials of SAARDs will not be subject to errors resulting from circadian variation, whereas clinical measurements should be performed at the same time of day throughout the trial.

The authors wish to thank Miss K. P. Hinchcliffe for technical assistance, and Mrs R. H. Schofield for typing the manuscript.

The Clinical Pharmacology Unit is grateful to Roche Products Ltd for financial support. Mrs N. G. Sitton is supported by a grant from the North Yorkshire Area Health Authority.

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