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DETERMINATION OF PLATELET-MEGAKARYOCYTE REGENERATION TIME IN PAINTERS OCCUPATIONALLY EXPOSED TO ORGANIC SOLVENTS

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SUMMARY

An improved method for the determination of the regeneration time of human platelets is presented. The platelet-megakaryocyte regeneration times of painters exposed to organic solvents (n = 5; age range 23—48 years) were compared with a reference group (n = 5; age range 23—46 years) not occupationally exposed to solvents. After purification and extraction of the samples, they were analysed by high-performance liquid chromatography. The results showed that the painters have a shorter regeneration time (range 4.4—5.2 days) than the reference group (range 5.2—6.9 days) and indicate that solvent exposure may be associated with an increase in platelet turnover in man.

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INTRODUCTION

Human platelets contain the enzyme fatty acid cyclo-oxygenase. Arachidonic acid can be transformed by this enzyme into several metabolites, one of which, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT), was analysed by high-performance liquid chromatography (HPLC) [10].

Fatty acid cyclo-oxygenase is irreversibly inhibited by a single oral dose of acetylsalicylic acid (ASA) [1]. Thus only newly formed platelets from the bone marrow will contain an active enzyme. By monitoring the induced maximal production of 12-HHT with time, a measure of the platelet regeneration time is obtained [2—4].

Studies on the impact of long-term exposure to organic solvents on humans have shown a higher kinetic uptake of serotonin in platelets compared with those of a reference group [5, 6]. Organic solvents have also been shown to cause functional changes in the central nervous system [7—9]. It was therefore of interest to compare the regeneration time of the platelets from solvent-exposed painters with corresponding data from a reference group not occupationally exposed to solvents.

EXPERIMENTAL

Preparation of internal standard

The internal standard (13-L-hydroxy-6,9,11-octadecatrienoic acid; 13-HOA) was prepared as described by Hamberg [11], modified as outlined below. The enzyme soyabean lipo-oxygenase (lipoxidase, EC 1.13.11.12), activity 20 000 U mg/or higher (Sigma, St. Louis, MO, U.S.A.), stored at 0°C, was incubated with the substrate, γ-linolenic acid (6,9,12-octadecatrienoic acid) (Sigma), at 0°C and pH 10.5. The substrate (4 mg) was converted into its ammonium salt by addition of 1 ml of ammonia solution (0.019 mol/l). The enzyme was dissolved in borate buffer [1 mg of soyabean lipo-oxygenase per ml of borate buffer (pH 10.5)].

Incubation. To the substrate solution were added 2.5 ml of the enzyme solution, followed by an additional 0.5 ml of enzyme after 15 min. UV absorbance measurement (234 nm) showed that the reaction was completed after 30 min. Ethanol (95%, for UV spectroscopy) was added (15 ml) and the reaction mixture was immediately diluted with distilled water (170 ml), acidified with hydrochloric acid (2 mol/l) to pH 3 and extracted with diethyl ether (ca. 100 ml). The ether phase was washed with distilled water to pH 7 and evaporated to dryness (Rotavapor) at room temperature.

The residue was dissolved in absolute ethanol (2 ml) and mixed with a solution of tin(II) chloride (15 mg) in absolute ethanol (0.5 ml). After 5 min at room temperature the solution was diluted with distilled water (100 ml) and acidified with hydrochloric acid (2 mol/l) to pH 2, extracted with diethyl ether (3 × 60 ml) and evaporated to dryness.

The residue was dissolved in *n*-hexane (Rathburn Chemicals; HPLC grade) and purified by preparative liquid chromatography. The preparative column and the mobile phase were the same as used later in the HPLC analysis. The

concentration of the internal standard was 19 ng/ml in 95% ethanol (for UV spectroscopy), as determined spectrophotometrically using an ϵ value of 30 000 (236 nm). The internal standard was stored at −20° C.

Blood donors

Five male painters (age range 23—48 years) were investigated with regard to haematological parameters. These workers were occupationally exposed to different types of industrial solvent mixtures that occur in paint. They had been employed in this work for 6—12 years.

The reference group consisted of five healthy volunteers (age range 23–46 years).

Blood sampling

No intake of acetylsalicylic acid by the subjects was allowed later than 10 days before blood sampling. Blood sampling took place at 8.00 a.m. after the test subjects had been fasting for 8 h. Blood (3 × 10 ml) was withdrawn from the test subjects using a Wasserman cannula ("wing-needle", I.D. 1.2 mm) and collected into three 15-ml plastic centrifuge test-tubes each containing 0.8 ml of 0.077 mol/l Na_2EDTA solution. Then acetylsalicylic acid (750 mg) (Dispril; Meda, Sweden) was administered to the subjects together with half a glass of water.

Blood samples were taken from the test subjects on five consecutive occasions: Monday (day 0), Wednesday (day 2), Friday (day 4), Monday (day 7) and Wednesday (day 9).

Preparation of platelets from blood

The blood was centrifuged for 12 min (200 *g*) and the plasma was transferred into two plastic centrifuge test-tubes (15 ml) with a plastic-tipped pipette (Selectapette, Clay Adams; Becton, Dickson, Parsippany, NY, U.S.A.). Plasma closer than 0.5 cm above the red blood cells was not collected, as the presence of red blood cells should be avoided in the plasma. Two 50-μl samples of plasma were taken for determination of the platelet concentration (Linson 431 A). Each 50-μl sample was diluted with 2.0 ml of isotonic saline (Baker).

Electronic cell counting was conducted in a Linson 431 A. The collected plasma was centrifuged for 12 min (650 *g*) and the plasma layer was removed with a pipette. A white platelet mass was now visible in the bottom of the test-tubes, to which were carefully added 3 ml of Tris—EDTA solution (8 ml of Tris—HCl buffer, 0.15 mol/l, pH 7.4; 2 ml of Na_2EDTA, 0.077 mol/l; 90 ml of sodium chloride, 0.15 mol/l; 0.2 g of glucose). The test-tubes were carefully rotated and the supernatant was removed. After addition of 5 ml of Tris—EDTA to each test-tube, the platelets were carefully suspended with a pipette fitted with a plastic tip.

The suspensions were centrifuged for 12 min (650 *g*). The washing procedure was repeated once, and the sample was transferred into a plastic centrifuge test-tube (15 ml) and centrifuged for 12 min (650 *g*).

After the removal of the Tris—EDTA solution, 1.1 ml of Tris solution (8 ml of Tris—HCl buffer, 0.15 mol/l, pH 7.4; 92 ml of sodium chloride, 0.15 mol/l; 0.2 g of glucose) was added. The platelets were carefully suspended with

a plastic-tipped pipette so that a homogeneous suspension was obtained without the platelets clustering together.

Two 50-μl samples were taken for the determination of the platelet count, both being diluted (1:6) with 250 μl of Tris solution. From each of these suspensions, 50 μl were withdrawn and diluted with 2.0 ml of isotonic saline (Baker). Electronic cell counting was carried out, the registered value being multiplied by 6 in order to obtain the accurate platelet count.

The suspension was diluted to 500 000 platelets per microlitre. The volume of Tris solution needed to obtain this platelet count was calculated in the following way: to 1 ml of suspension, x ml of Tris solution were added:

$$x = \frac{(\text{platelet count} \times 6) - 500\,000}{500\,000}$$

After the platelet count was verified, 1.5 ml of the diluted suspension were transferred into a plastic centrifuge test-tube (15 ml) in which was placed a small PTFE-coated magnet. The test-tube was placed in the 37°C water-bath and the magnetic stirrer was started.

Incubation of platelets with arachidonic acid

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) (Sigma) was dissolved in 95% ethanol (for UV spectroscopy) to give a 25 mg/ml solution, which was stored under nitrogen. From this batch, 1.8 μl were withdrawn with a Hamilton syringe (corresponding to 45 μg of arachidonic acid). When the test-tube had been heated in the water-bath for 2 min, the arachidonic acid was added. After 10 min, the reaction was stopped by the addition of 8.5 ml of 95% ethanol (for UV spectroscopy). Magnetic stirring was continued for a further 1 min. A 500-μl volume of the internal standard was then added. This addition gave a peak of the internal standard on the HPLC trace that was of the same size as the 12-HHT peak. At this stage, it was possible to store the sample at −20°C for 1−2 days.

Extraction of 12-HHT

The sample was transferred into a beaker (100 ml), diluted with 50 ml of distilled water and acidified with hydrochloric acid (2 mol/l) to pH 2.3 (ca. 4 drops). The pH was checked with a pH meter.

The sample was then extracted twice in a separating funnel with 60 ml of diethyl ether. After the first extraction, the upper ether layer was collected and the water layer, sometimes containing an emulsion, was extracted once again with diethyl ether. The combined ether layers were washed with 3 × 15 ml of distilled water until the pH was 5.5.

The ether was evaporated at 30°C (water-bath). When most of the diethyl ether had evaporated, the temperature was raised to 35°C and the evaporation was continued until the remaining water had evaporated (ca. 10−30 min). The sample was dissolved in 1 ml of acetone and transferred into a 10-ml glass centrifuge test-tube; this was repeated once with 1 ml of acetone. The test-tube was placed in a water-bath (30°C) and the acetone was evaporated under nitrogen (it was of the utmost importance that the test-tube was completely

dry; this could take ca. 1 h). If some water remained in the test-tube, it was possible to remove it from the residue with acetone which was then evaporated under nitrogen. The dry sample was dissolved in 0.2 ml of *n*-hexane (HPLC grade), and centrifuged for 10 min at 1370 *g*. The sample was then transferred with a Pasteur pipette into a small glass tube (disposable borosilicate glass culture tubes, 75 × 10 mm; Kimble, Owens, IL, U.S.A.). The sample could then be stored under nitrogen at −20°C.

High-performance liquid chromatography

The extracts were analysed by HPLC (Perkin-Elmer Series 3B chromatograph) equipped with a Perkin-Elmer LC-85 spectrophotometric detector, set at 235 nm. The apparatus was equipped with an integrator. A 20-μl loop was installed in the sample injector (Rheodyne). The stainless-steel analytical column (100 × 4.6 mm I.D.) was packed with Nucleosil 50-5, particle size 5 μm (Macherey-Nagel, Düren, F.R.G.). Metal filters with diameters of 4.6 mm (2 μm) were fitted at both ends.

A pre-column (50 × 4.6 mm I.D.) was filled (not packed) with the same stationary phase and equipped with similar filters at both ends. This pre-column was installed between the HPLC pump and the sample injector in order to equilibrate the mobile phase.

The mobile phase consisted of *n*-hexane—isopropanol—acetic acid (98.4: 1.6:0.04). Air bubbles were removed from the mixture under vacuum and using an ultrasonic water-bath.

Before the start of the analysis, *n*-hexane was injected in order to check the baseline. Then, a sample of the internal standard was injected to verify the retention time of the internal standard. The peak height and area of the internal standard were compared with those from previous analyses, to check its stability after storage. Each sample was analysed at least twice and the average of the sample peak area divided by the internal standard peak area was calculated. Peak areas could also be approximated by multiplying the peak height by the peak width at half-height.

Calculation of the platelet regeneration time

The cyclo-oxygenase activity for sample No. 1 (day 0) was set to 100% on the *y*-axis. Values for samples Nos. 2—5 (days 2—9) were each divided by the value for No. 1 and plotted against the number of days. When 50% of the original cyclo-oxygenase activity had been regained, the regeneration time was read off the *x*-axis. An Apple IIe computer, together with Scientific Plotter software (Interactive Microware, State College, PA, U.S.A.), was used. Statistical analysis of the difference between the exposed and the reference group was performed using the Wilcoxon rank sum test (NPAR1WAY Procedure, SAS Institute, Cary, NC, U.S.A.).

RESULTS

Platelet regeneration times for the exposed painters and the reference group are shown in Table I. The regeneration time was significantly lower in the exposed group ($p < 0.02$). The range for the controls was 5.20—6.95 days

(mean 6.02 days) and that for the exposed individuals was 4.35—5.17 days (mean 4.77 days).

TABLE I

PLATELET REGENERATION TIME: MEANS, STANDARD DEVIATIONS AND WILCOXON TWO-SAMPLE TEST RESULTS (Z, P)

Exposed subjects		Reference subjects	
No.	Regeneration time (days)	No.	Regeneration time (days)
E1	4.44	R6	6.03
E2	4.35	R7	5.54*
E3	5.17	R8	6.39
E4	5.03	R9	6.95
E5	4.89	R10	5.20**
Mean	4.77	Mean	6.02
S.D.	0.32	S.D.	0.62

$Z = 2.5067$
$P = 0.0122$

*Exposed to minor amounts of organic solvents.
**Exposed to organic solvents in his leisure time.

DISCUSSION

This technique described here is considerably faster and more convenient than previous techniques using thin-layer chromatography [2—4]. The analysis time is approximately 10 min and the HPLC peaks can be automatically quantified using an electronic integrator. The platelet regeneration time was determined from the HPLC analysis of the cyclo-oxygenase metabolite 12-HHT, compared with an internal standard, 13-L-hydroxy-6,9,11-octadecatrienoic acid (13-HOA). The extraction and the chromatographic procedures were controlled for possible interferences by analysis of different blank runs.

Examples of HPLC traces are shown in Figs. 1—3.

Fig. 1a shows the chromatogram from a blank (1.5 ml of Tris solution) free from platelets, incubated for 10 min without the addition of arachidonic acid. Internal standard (500 μl) was added before the sample was extracted with diethyl ether. Fig. 1b shows the chromatogram from a non-exposed blood donor, after analogous treatment. A 12-HHT peak was not observed, which shows that no interfering substances were present. A platelet suspension from the same blood donor as in Fig. 1b was incubated with arachidonic acid for 10 min. Internal standard (500 μl) was added before the extraction. A 12-HHT peak was present in the chromatogram obtained (Fig. 1c).

Fig. 2b shows the chromatogram of a sample from a member of the reference group obtained on day 0, before ASA intake, and Fig. 2a shows the chromatogram of a sample from the same subject obtained on day 2, after ASA intake. The 12-HHT level shown in Fig. 2a is considerably lower than the corresponding level in Fig. 2b.

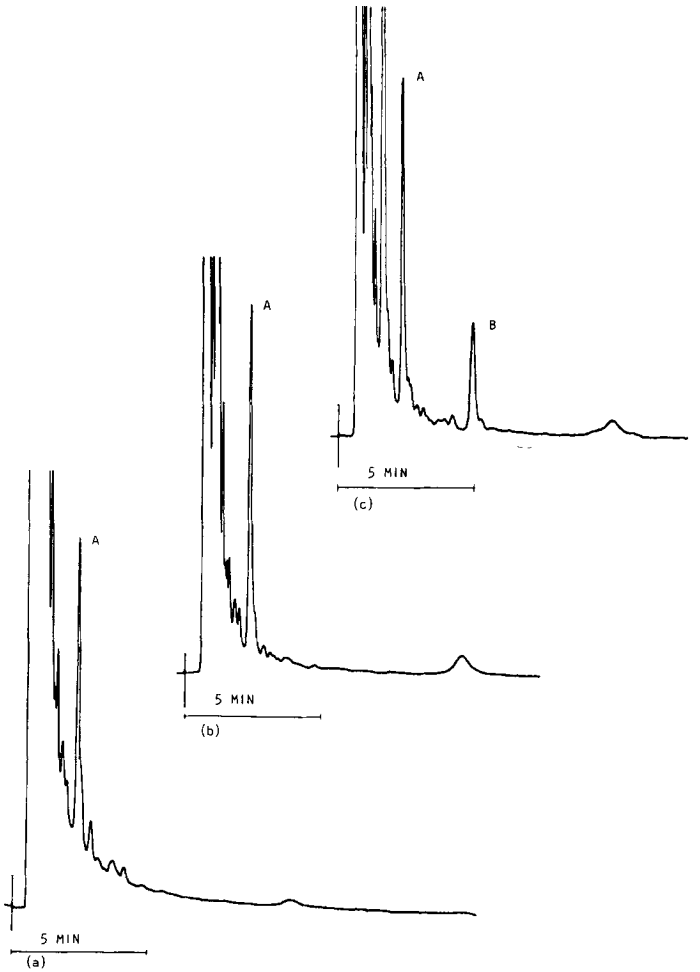

Fig. 1. Chromatograms of three blanks, each with 500 μl of internal standard added before extraction with diethyl ether and each incubated for 10 min. (a) 1.5 ml of Tris solution, free from platelets and arachidonic acid. (b) A platelet suspension from a blood donor, free from arachidonic acid. (c) A platelet suspension from the same blood donor as in (b) but with the addition of arachidonic acid. Peaks: A = 13-HOA; B = 12-HHT.

Fig. 3 shows the chromatograms of samples from an exposed painter, obtained on (b) day 0, before ASA intake, and (a) day 2, after ASA intake. The 12-HHT level in Fig. 3a is much lower than the corresponding level in Fig. 3b.

The general health of the subjects in the exposed group was similar to that of those in the reference group. Background variables such as smoking and drinking habits were similar. In the reference group, subjects 7 and 10 were at least to some degree exposed to organic solvents in their daily work. Subject 7 works part time in a chemical laboratory and subject 10 full time in a pharmaceutical factory. The subject (10), who exhibited the shortest regeneration time (5.2 days) was interviewed again. It was than revealed that during

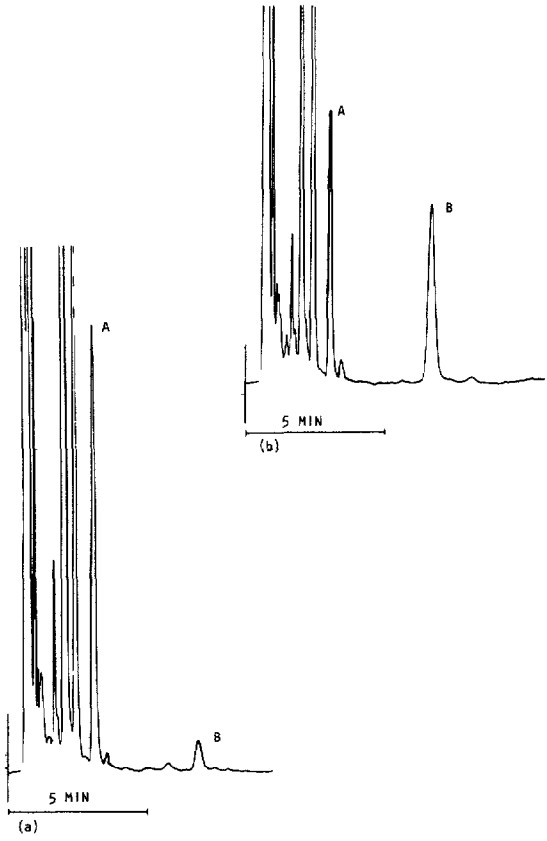

Fig. 2. Chromatograms of samples from a member of the reference group, obtained (b) on day 0 (before ASA intake) and (a) on day 2 (after ASA intake). Peaks: A = 13-HOA; B = 12-HHT.

a 6-month period, just before the sampling of blood, he had been building a large plastic sailboat-form in his leisure hours, and had obviously received a higher degree of exposure to organic solvents. Thus the platelet regeneration time for subject 10 was similar to the level found for the exposed group. Subject 7 had been a regular blood donor for several years, which also might affect the regeneration time of the platelets.

The platelet regeneration time was determined by plotting the enzyme activity, obtained from the HPLC data, against time (days). The regression line was calculated using an Apple IIe microcomputer (Figs. 4 and 5).

Fig. 4 shows the fitted curve of the values from a member of the reference group (enzyme activity versus regeneration time). After ingestion of ASA, there is a two-day lag phase in the platelet prostaglandin synthesis, via the cyclo-oxygenase pathway, attributed to acetylation of the megakaryocyte cyclo-oxygenase [12]. The fact that the regression line does not pass through the origin can be explained by the assumption that the intake of ASA has a residual inhibitory effect on the reproduction of platelets, extending over several hours.

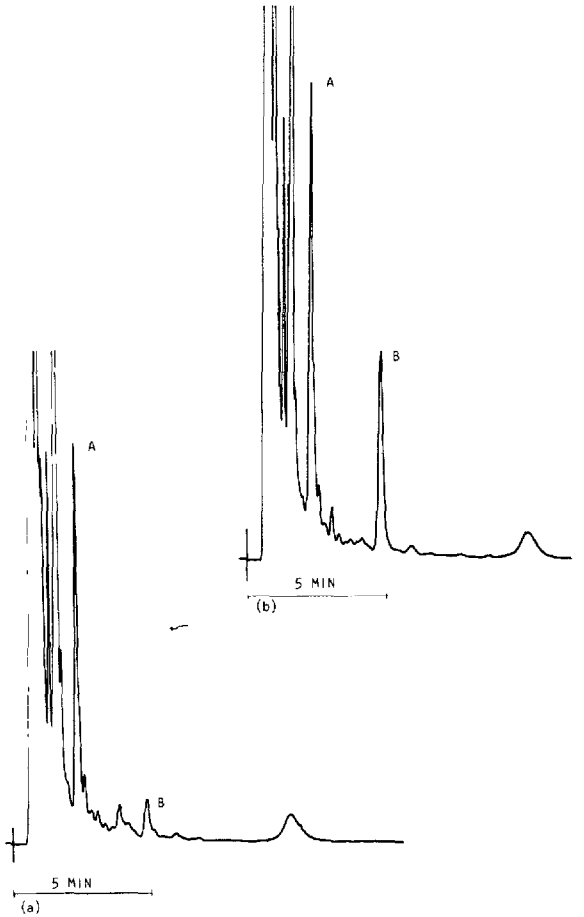

Fig. 3. Chromatograms of samples from an exposed painter, obtained (b) on day 0 (before ASA intake) and (a) on day 2 (after ASA intake). Peaks: A = 13-HOA; B = 12-HHT. The differences in retention times of 13-HOA and 12-HHT between the chromatograms of the reference subject (Fig. 2) and the exposed subject (Fig. 3) are due to a replacement of HPLC columns between those runs.

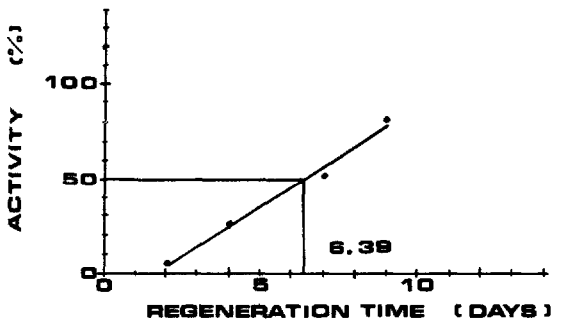

Fig. 4. Fitted curve (enzyme activity versus regeneration time) for a member of the reference group (R8).

Fig. 5 shows the fitted curve of the values from an exposed painter. The assumed inhibitory effect caused by remaining ASA is less evident here than for the non-exposed reference subject. (Fig. 4).

In earlier studies [5, 6], we observed changes in platelet function in workers exposed to organic solvents. The shortened platelet-megakaryocyte regeneration time observed in this study may be due to effects of the inhaled solvent mixtures. The results confirm our hypothesis [6] that the solvents may act directly on platelet-megakarocyte formation in the bone marrow.

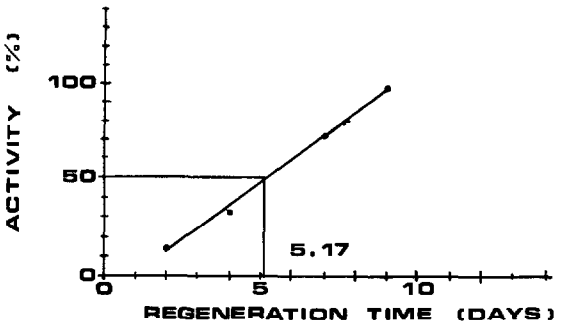

Fig. 5. Fitted curve (enzyme activity versus regeneration time) for an exposed painter (E3).

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