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## Purine enzyme activities in peripheral blood mononuclear cells: comparison of a new non-radiochemical high-performance liquid chromatography procedure and a radiochemical thin-layer chromatography procedure

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### Abstract

Purine enzyme activities are usually assayed by radiochemical procedures and often TLC is part of the separation method. In screening patients with rheumatic diseases, these procedures have shown disadvantages like a relatively large coefficient of variation (C.V.) and time-instability. We describe a non-radiochemical reversed-phase HPLC micro-method with UV detection for measurement of activities of purine 5'-nucleotidase (5'NT; EC 3.1.3.5), purine nucleoside phosphorylase (PNP; EC 2.4.2.1) and hypoxanthine guanine phosphoribosyltransferase (HGPRT; EC 2.4.2.8) in human peripheral blood mononuclear cells (PBMC). The HPLC procedure is compared with the radiochemical TLC procedure by testing both with a 5'NT and a PNP assay. Reproducibility is tested with 14 healthy controls in each procedure. Short-term and long-term time-stability is tested by comparing enzyme activities measured immediately after preparation of the PBMC (week 0) with those found after freezing and storage at  $-20^{\circ}\text{C}$  for a maximum of 10 weeks. The HPLC procedure is preferable to the radiochemical TLC procedure because it shows significantly better reproducibility and better time-stability and in addition is non-radiochemical and less time-consuming.

### 1. Introduction

Purine enzymes play an important role in physiological as well as in pathological conditions. Several immunodeficiencies are related to abnormalities of purine enzyme activities [1–7]. Impairment of purine enzyme activities is also

found in autoimmune diseases like autoimmune haemolytic anaemia [8,9], systemic lupus erythematosus [10] and rheumatoid arthritis (RA) [11]. Recently new data were reported concerning purine enzyme levels in RA patients treated with azathioprine (AZA) [12].

AZA is an immunosuppressive and cytotoxic agent which, after conversion to 6-mercaptopurine, is metabolized by purine enzymes. It is

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used in the treatment of a number of autoimmune diseases and malignancies and to prevent allograft rejection in organ transplant recipients. The outcome of AZA treatment may be strongly influenced by deficiencies of purine enzymes: HGPRT deficiency causes resistance to AZA treatment [13] and both thiopurine methyltransferase deficiency and low levels of 5'NT result in increased susceptibility to severe bone marrow depression during AZA treatment [14–17].

Purine enzyme activities are often measured by radiochemical procedures in combination with TLC separation. During a recent screening of purine metabolism in patients with RA, we found some distinct disadvantages of these procedures. Firstly, time-stability of enzyme activities of the freeze-dried PBMC stored at  $-20^{\circ}\text{C}$  was not as expected. Secondly, the C.V. was relatively large. Thirdly, regarding environmental protection, radiation hygienics and general applicability, radiochemical assays are out of favour nowadays, and finally, these procedures are rather time-consuming.

According to a Medline literature search starting from 1982 there are no previous reports comparing a radiochemical TLC procedure with a non-radiochemical HPLC procedure to measure activities of the purine enzymes 5'NT and PNP, nor reports of HPLC procedures to measure purine enzyme activities of 5'NT, PNP or HGPRT in human PBMC.

In this paper we present a new non-radiochemical reversed-phase HPLC micro-method which obviates the above-mentioned disadvantages. Reproducibility and time-stability were tested with PNP and 5'NT assays using both procedures. Time-stability of the HPLC procedure was also tested with an HGPRT assay. Tests were carried out with blood samples from healthy volunteers.

## 2. Experimental

### 2.1. Design of the studies

To determine reproducibility each procedure was tested with PBMC of 14 different healthy

volunteers. The C.V. was considered for each person. Mean age was 51.1 years (range: 43–64) in the radiochemical TLC group and 44.3 years (28–64) in the HPLC group.

Time-stability was tested with PBMC of 5 and 3 healthy persons respectively for the radiochemical TLC procedure and the HPLC procedure. The mononuclear cells were isolated only once, prepared according to each procedure and kept in stock in a number of Eppendorf tubes (one tube for each single measurement). For the radiochemical TLC procedure enzyme activity measurements of PNP and 5'NT were done the day after isolation of the PBMC (week 0) and at weeks 1, 2, 3, 6 and 8. The testing of the PNP, 5'NT and HGPRT stability by the HPLC procedure was done at weeks 0, 1, 2, 8 and 10.

All enzyme assays were carried out in quadruplicate.

### 2.2. Isolation of mononuclear cells from peripheral blood

A 10-ml volume of venous blood was defibrinated (on glass beads) as soon as it was obtained and immediately processed for further analysis. An equal volume of 0.9% (w/v) NaCl (NPBI) was added and 8 ml of this suspension was carefully layered on top of a density gradient, containing 4 ml Ficoll-Isopaque (density 1.077 g/ml, Nycomed). Centrifugation (Sorvall RC3, DuPont Company, Newtown, CT, USA) was carried out at room temperature for 20 min at 1000 g. PBMC were collected from the interface, washed with approximately the same volume of 0.9% NaCl and centrifuged (Hettich Universal 30 RF, Hettich Zentrifugen, Tuttlingen, Germany) at  $20^{\circ}\text{C}$  for 5 min at 825 g. The contaminating erythrocytes were eliminated by resuspending the cell pellet in a 10-ml erythrocyte shock solution consisting of 170 mM Tris (Merck) and 155 mM  $\text{NH}_4\text{Cl}$  (Merck) (1:9, v/v) (pH 7.2–7.4). The suspension was incubated for 15 min in a water-bath at  $37^{\circ}\text{C}$  (Julabo SW-20C, Julabo Labortechnik, Seelbach, Germany) and centrifuged again at  $20^{\circ}\text{C}$  for 5 min at 825 g. After this, the cell pellet was resuspended and

washed twice in approximately 15 ml of 0.9% NaCl and centrifuged after each washing step at 20°C for 5 min at 825 g. Then the pellet was resuspended in 1.25 ml of a solution of 0.9% NaCl containing 4% bovine serum albumin (Organon Teknika, Boxtel, Netherlands) and temporarily stored on ice. Numbers of white blood cells were determined by a Coulter Counter (Model S5, Coulter Electronics, Luton, UK). Mononuclear cell viability, monitored by staining with trypan blue, was  $94.8 \pm 2.9$  (% mean  $\pm$  S.D.). Finally, for each enzyme assay an exact number of mononuclear cells, corrected for viability, was collected in Eppendorf tubes.

The PNP assay requires 1500 cells for the HPLC procedure and 500 cells for the radiochemical TLC procedure, the 5'NT assay 18 000 and 6000 cells respectively and the HGPRT assay 15 000 cells for the HPLC procedure.

The cells were frozen at  $-70^\circ\text{C}$  and lyophilized overnight (Lyovac GT2, Leybold, Köln, Germany). For the radiochemical TLC procedure the pretreated cells were stored at  $-20^\circ\text{C}$  until the enzyme assay was carried out. For the HPLC procedure, the enzyme assays were performed the next day and the supernatants were stored at  $-20^\circ\text{C}$  until the HPLC separation.

### 2.3. The radiochemical TLC procedure

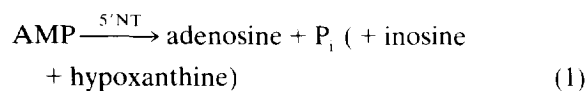
The enzyme assays, the TLC separation methods and the calculation of specific enzyme activities were carried out essentially according to procedures published previously [18].

### 2.4. The non-radiochemical HPLC procedure

#### Enzyme assays

##### Purine-5'-nucleotidase.

5'NT activity was determined from degradation of adenosine monophosphate (AMP) to adenosine:

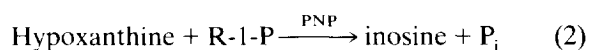


A reaction mixture with a pH of 8.5 was used,

consisting of 8 nmol/10  $\mu\text{l}$  AMP (Sigma), 225 mM Tris (Merck) + 90 mM  $\text{MgCl}_2$  (Merck) + 1.875 mM  $\beta$ -glycerolphosphate (Sigma) and 10% Triton X-100 (Sigma) (10:20:1, v/v). A 10- $\mu\text{l}$  volume of the reaction mixture was added to the lyophilized PBMC. After 3 h of incubation at 37°C, the reaction was stopped by addition of 10  $\mu\text{l}$  of 0.8 M ice-cold perchloric acid (PCA) (Merck). The deproteinized incubation mixture was centrifuged at 4°C and 17 000 g for 4 min. After centrifugation, 15  $\mu\text{l}$  of the supernatant was neutralized with 35  $\mu\text{l}$  of 0.2 M  $\text{K}_2\text{HPO}_4$  (Merck). The neutralized mixture was centrifuged again and the supernatant was stored at  $-20^\circ\text{C}$ .

##### Purine nucleoside phosphorylase.

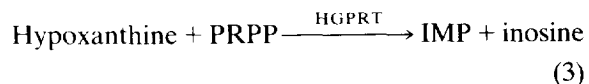
PNP activity was determined from the conversion of hypoxanthine to inosine:



The reaction mixture had a pH of 7.0 and consisted of 4 nmol/10  $\mu\text{l}$  hypoxanthine (Sigma), 5 mM ribose-1-phosphate (R-1-P) (Sigma), 0.5 M Tris + 10 mM EDTA (Siegfried S.A.) and 10% Triton X-100 (10:10:10:1, v/v). Of this mixture 20  $\mu\text{l}$  were added to the lyophilized PBMC. The reaction was allowed to proceed for 1 h at 37°C. The incubation mixture was deproteinized, neutralized and stored according to the method used for the purine 5'-nucleotidase assay.

##### Hypoxanthine-guanine phosphoribosyltransferase.

HGPRT activity was determined from the conversion of hypoxanthine to inosine monophosphate (IMP):



A reaction mixture with a pH of 7.4 was used, consisting of 4 nmol/10  $\mu\text{l}$  hypoxanthine (Sigma), 8 mM phosphoribosyl pyrophosphate (PRPP) (Sigma), 0.5 M Tris + 0.05 M  $\text{MgCl}_2$  and 10% Triton X-100 (10:10:10:1, v/v). Twenty microliters of this mixture were added to the

lyophilized PBMC. The reaction was allowed to proceed for 1.5 h at 37°C. The incubation mixture was deproteinized, neutralized and stored according to the method used for the purine 5'-nucleotidase assay.

#### HPLC separation method

An automated HPLC system (LKB, LKB-Produkt, Bromma, Sweden) was used consisting of an autosampler (20- $\mu$ l sample loop) (Waters 717 Autosampler, Millipore Corporation, Marlborough, MA, USA), a Supelcosil LC-18-S HPLC column (250  $\times$  4.6 mm I.D.; particle size 5  $\mu$ m) (Supelco, Bellefonte, PA, USA), a 2150 HPLC pump (LKB), a continuous mobile-phase degasser (LKB 2156 solvent conditioner) and a UV detector with wavelength set at 254 nm (Applied Biosystems 785 A programmable absorbance detector, Applied Biosystems, Foster City, USA). Automatic data capture and processing were performed by Millennium software (Millipore-Waters, USA). The operating pressure was 90 bar and the flow-rate was 1.25 ml/min. A two-buffer system gradient, buffer A [0.025 M  $\text{KH}_2\text{PO}_4$  (Merck)] and B [25% methanol (Lab-Scan)–75% 0.05 M  $\text{KH}_2\text{PO}_4$ ], was

used for separation (Fig. 1). The method previously published [19] was modified slightly.

The incubation mixture stored at  $-20^\circ\text{C}$  was thawed and centrifuged at  $20^\circ\text{C}$  for 4 min at 17 000 g. Twenty microliters of the supernatant were injected onto the HPLC system.

Control preparations consisted of two blanks, the reaction mixture (A) and a combination of reaction mixture plus isolated enzyme without substrate (B), and three standard solutions. For each type of enzyme assay a specific own blank A was analyzed. A blank B was analyzed with the HPLC procedure for each enzyme assay per person. The three standard solutions were measured per ten samples. The standard solutions contained all six purine nucleotides and nucleosides, each of them in three concentrations.

#### Calculation of specific enzyme activity

For each substrate and each product a standard curve of the peak area against the concentration was made. After separation, the concentration of the products could be determined from their area under the curve value. The specific enzyme activity was calculated and expressed in  $\text{nmol}/10^6$  viable cells/h.

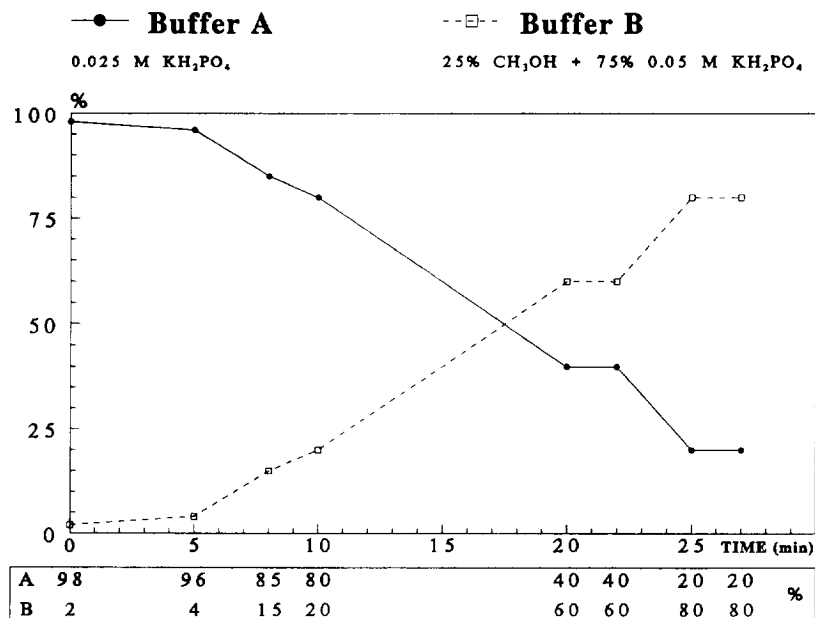


Fig. 1. Gradual change of proportions of the two-buffer system during HPLC separation.

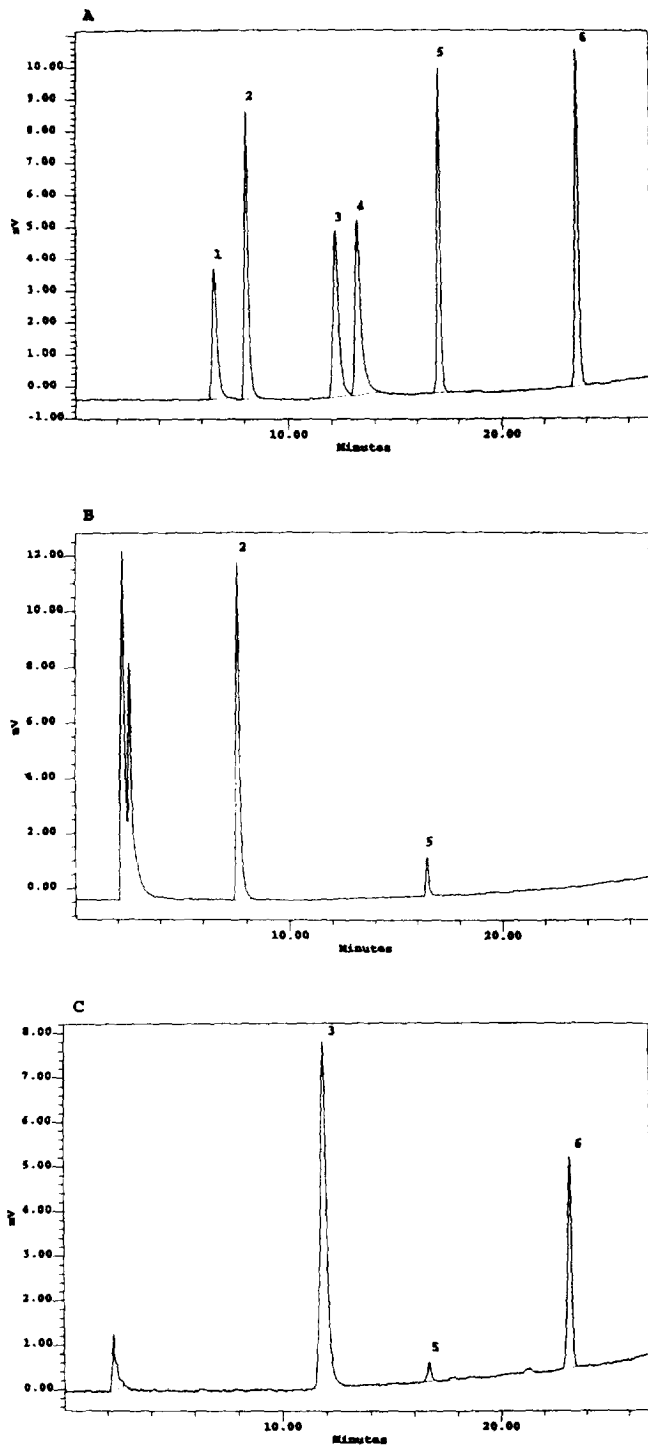


Fig. 2.

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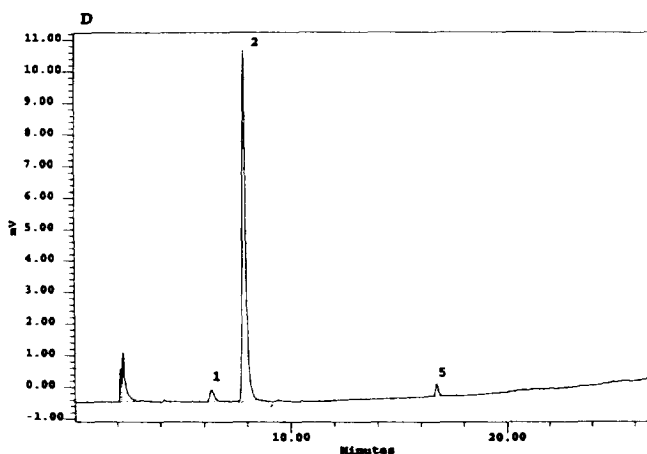


Fig. 2. Chromatograms of (A) a standard mixture of nucleosides and mononucleotides, each at a concentration of  $10 \mu\text{M}$ ; (B) PNP purine enzyme assay; (C) 5'NT purine enzyme assay and (D) HGPRT assay. Peaks: 1 = IMP, 2 = hypoxanthine, 3 = AMP, 4 = adenine, 5 = inosine, 6 = adenosine. Chromatography was performed under the conditions described in the text.

### 2.5. Statistics

To analyze the reproducibility of both the radiochemical TLC and the HPLC procedure, the C.V. for the PNP assay and the 5'NT assay was calculated from the data-set of each control subject. The differences between the procedures, regarding the mean C.V., were examined by using the non-parametric two-sided Wilcoxon rank-sum test.

Differences in time-stability were analyzed on the basis of a fixed two-way analysis of variance (ANOVA) model with fixed patient effects and a mixed two-way ANOVA model with random patient effects. Additionally pairwise differences between times were analyzed further according to the multiple comparison method of Scheffé [20].

The statistics were processed by SAS computer software (SAS Institute, Cary, NC, USA) and by a SAS macro. Testing results were considered significant if  $p \leq 0.05$ .

### 3. Results

Examples of HPLC profiles of a standard mixture of nucleosides and mononucleotides and of the substrates and products from the enzyme

assays are shown in Fig. 2. Only negligible amounts of product (inosine in the PNP assay, adenosine in the 5'NT assay and IMP in the HGPRT assay) were present in the chromatograms of the mononuclear cell suspensions incubated without substrate (data not shown).

Under the conditions mentioned before, the enzyme assays of the HPLC procedure were tested for linearity between product formation (enzyme activity) and number of cells used for the assay. Per enzyme each data point was assayed in triplicate. The results are shown in Fig. 3.

Experiments by HPLC with standard nucleotide and nucleoside solutions of IMP, AMP, inosine, adenosine and hypoxanthine, each in three known concentrations, showed a mean recovery  $\pm$  S.D. of  $103.7 \pm 7.2$ ,  $93.7 \pm 3.6$ ,  $94.6 \pm 7.2$ ,  $94.3 \pm 6.1$  and  $92.8 \pm 5.9\%$ , respectively.

Analysis of the reproducibility of PNP activity and 5'NT activity measured by both methods was carried out as described above. All procedures were performed within the first two weeks after blood sampling. The results are given in Table 1. In both enzyme assays the C.V. of the HPLC procedure was significantly lower ( $p = 0.002$ ).

Time-stability was examined by measuring the

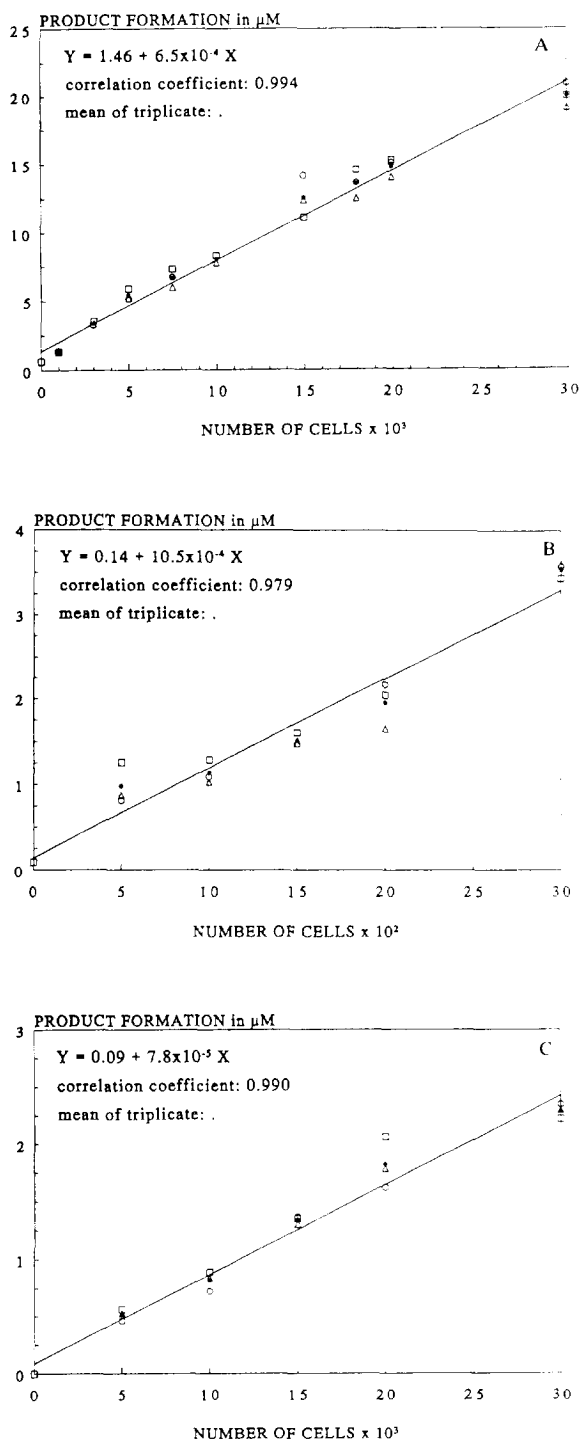


Fig. 3. Linearity between product formation and the number of cells used in the 5'NT (A), PNP (B) and HGPRT (C) assays (HPLC procedure).

enzyme activity at certain time intervals of either a sample of the pretreated and stored PBMC (radiochemical TLC procedure) or a sample of the stored supernatant of the deproteinized incubation mixture (HPLC procedure).

The 5'NT assay measured by the radiochemical TLC procedure (Fig. 4) is not stable in time. According to a fixed two-way ANOVA, averaged over the 5 persons, not all time means were equal ( $p < 0.001$ ). This is caused mainly by a significant decrease between week 0 and the subsequent weeks. During the following weeks there is a decreasing trend in averaged 5'NT enzyme activities. Examining the data on the basis of a two-way mixed ANOVA model with random patient effects, followed by a multiple comparison procedure of Scheffé (data not shown), the same conclusions could be drawn.

For PNP assayed by the radiochemical TLC procedure, the hypothesis of equal time means, averaged over the 5 persons, also has to be rejected ( $p < 0.001$ ). A nearly significant decrease was found between week 0 and weeks 1, 3, 6 and 8 (Fig. 4). However, at week 2, the enzyme activity is again at the level measured before freezing the PBMC. Under the mixed two-way ANOVA model (data not shown) the differences are somewhat less distinct but still point in the same direction.

As regards the 5'NT assay tested by the HPLC procedure, the hypothesis of equal time means, averaged over 3 persons, has to be rejected ( $p < 0.001$ ) too. No average differences were found between weeks 1, 2, 8 and 10 (Fig. 4), but enzyme activity in week 0 is significantly higher than during the following weeks. The mixed two-way ANOVA model with random patient effects, followed by a multiple comparison procedure of Scheffé (data not shown) gives the same result and also points to an effect of freezing.

The PNP assay measured by the HPLC procedure is very stable. On average all weeks have about the same enzyme activity ( $p > 0.10$ ) (Fig. 4).

Concerning the HGPRT assay, averaged over the 3 persons, the time means show no equality ( $p < 0.001$ ). However, only at week 8 a sig-

Table 1  
Coefficients of variation of the radiochemical TLC procedure and the HPLC procedure with the PNP and 5'NT enzyme assays

	PNP assay		5'NT assay	
	TLC (n = 14)	HPLC (n = 14)	TLC (n = 14)	HPLC (n = 14)
Mean C.V.	12.3	6.6	11.2	5.3
S.E.M.	1.4	0.8	1.5	0.9
<i>Percentiles</i>				
100%	27.8	14.4	20.3	13.5
50%	11.7	6.0	8.7	4.2
0%	5.1	3.2	2.9	2.0
<i>p</i> -Value <sup>a</sup>	0.002		0.002	

S.E.M.: standard error of the mean C.V.

<sup>a</sup> Wilcoxon 2-sample test.

nificantly higher mean is found compared with the other times ( $p \leq 0.05$ ) (Fig. 4). The two-way mixed ANOVA model showed no differences among all the weeks, indicating that the impairment at week 8 is only a minor effect, probably not important in studies testing larger groups.

Finally it is noted that for 5'NT and PNP assayed by the radiochemical TLC procedure and for HGPRT assayed by the HPLC procedure, the different persons do not show parallel time profiles (interaction tests of the fixed two-way ANOVA yielding  $p = 0.001$ ,  $p = 0.02$  and  $p = 0.02$  respectively).

#### 4. Discussion

Screening large numbers of patients for their purine enzyme activities often causes difficulties in planning and questions about feasibility because the laboratory procedures are rather complicated and time-consuming. These problems might be overcome if the laboratory procedure could be interrupted and the patient material temporarily stored without effect on the enzyme activities measured.

In this way the time stability of the radiochemical TLC procedure was tested when it was interrupted after isolation, purification, division and lyophilization of the PBMC. The results were disappointing, because of a gradual de-

crease of 5'NT enzyme activity. Possibly this was due to decrease of ecto-5'-nucleotidase activity. The localization on the outside of the cell membrane could make this enzyme more sensitive to manipulation of the cells than cytoplasmatic enzymes. It is obvious that freezing and storing at  $-20^{\circ}\text{C}$  of prepared mononuclear cells is not adequate to preserve 5'NT purine enzyme activity for a longer period of time. Therefore in our study design this radiochemical TLC procedure is not acceptable.

The PNP assay in the radiochemical TLC procedure shows a more stable enzyme activity during the 8 weeks with two exceptions. The higher enzyme activity at week 0 indicates a possible effect of freezing. No adequate explanation can be given of the significantly higher activity in week 2. It is very unlikely that enzyme activities return to pre-freezing levels. Although activity of week 2 seems to be significantly higher, it still falls within the range of the standard deviations, so these values may be coincidental and part of the normal fluctuations of this procedure.

The TLC procedure itself may also play an important role in the moderate results. The separation between substrate and products is not always very sharp and may cause minor errors when the several zones have to be cut out for fluid scintillation counting. By doing so another source of inaccuracy of the results is the crum-



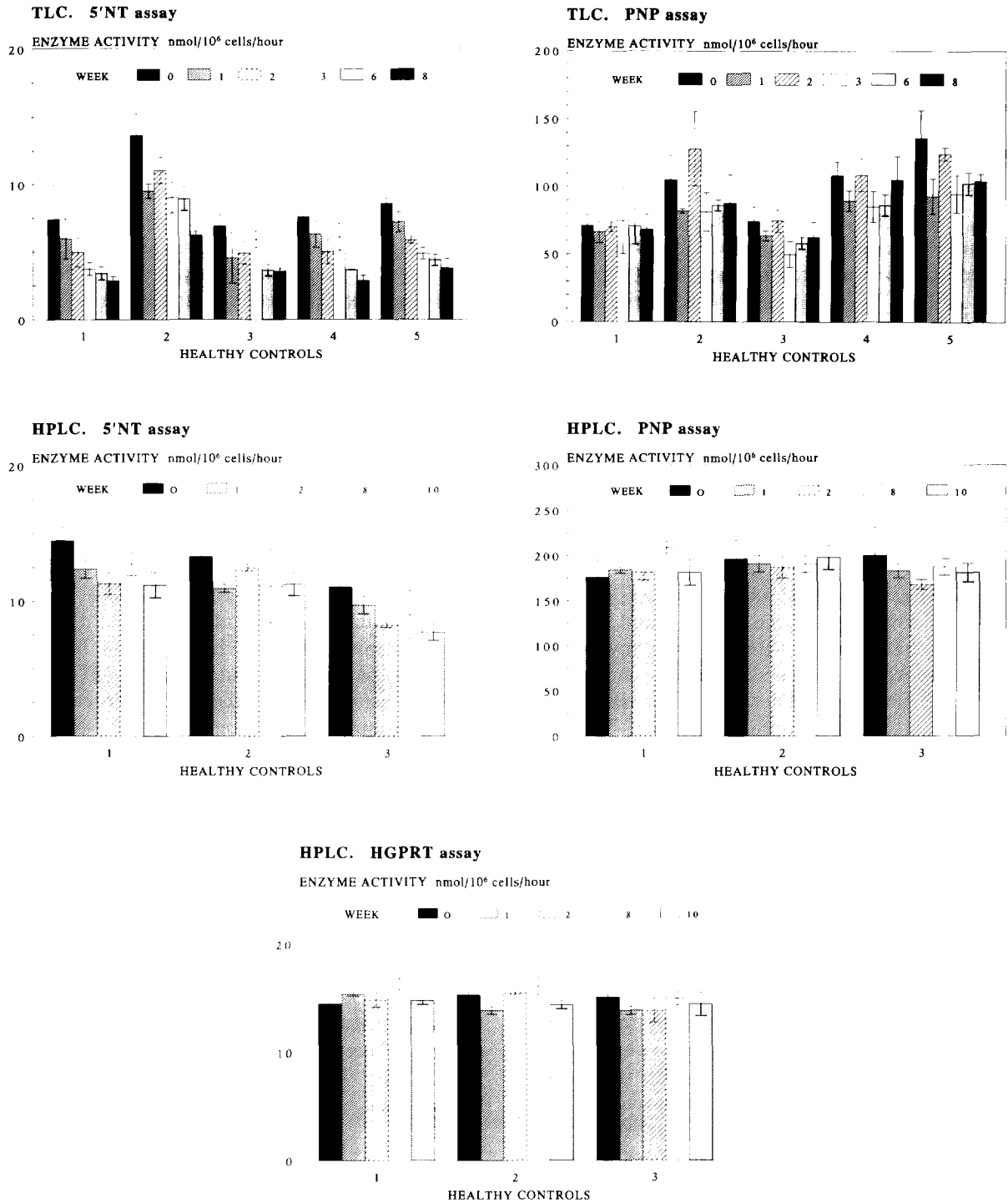


Fig. 4. Time-stability diagrams of the radiochemical TLC procedure and the HPLC procedure (means ± S.D.).

bling away of small fragments of the thin layer from the cellulose carrier. Several sprays used to prevent crumbling of the thin layer gave no marked improvement of the results.

The above-mentioned difficulties with the radiochemical TLC procedure led to development of non-radiochemical HPLC procedures with UV detection to measure the activities of the purine enzymes 5'NT, PNP and HGPRT.

Apart from the separation itself the three main differences between the radiochemical TLC and the HPLC procedure are, firstly, the larger number of cells used in the HPLC procedure while it still remains a micro-method; secondly, after lyophilization of the PBMC essential steps (storage, incubation and separation) have a different sequence in the HPLC procedure; and, thirdly, the incubation is terminated by protein denaturation instead of cooling on ice.

The enzymatic conditions of 5'NT, PNP and HGPRT in the HPLC procedure are largely based on those of the radiochemical TLC procedure. Each enzyme assay displays a good linearity between enzyme activity and a wide range of cell numbers. Results of recovery experiments were good as well, and a sharp separation of mononucleotides and nucleosides was achieved in a total run-time of 25 min.

The reproducibility of the HPLC procedure is significantly better than that of the radiochemical TLC procedure, for both the PNP assay and 5'NT assay. Smaller differences in purine enzyme activities will be significant sooner in this HPLC procedure and with respect to our studies of purine enzyme activities in patients with rheumatic diseases [12,17], this is an important advantage.

Time-stability of the HPLC procedure was tested in short-term and long-term follow-up. The test with the 5'NT assay shows a clear effect between week 0 and the other weeks, indicating that freezing at  $-20^{\circ}\text{C}$  of the supernatant has still a decreasing effect on 5'NT activity measured. Because this procedure rules out a freezing and storing effect on the enzyme itself the cause has to be located on the level of substrate (AMP) and/or product [adenosine, (inosine and hypoxanthine)]. Apart from the freezing and

storing effect on the enzyme itself this hitherto unnoticed influence on substrate and/or product may also play an additional role in the decreasing 5'NT activity assayed by the radiochemical TLC procedure.

Interestingly, the PNP assay measured by the HPLC procedure has a very good time-stability, in the short as well as in the long term.

HPLC time-stability tested for the HGPRT assay is good, with an unexpected exception in week 8. This is probably merely a coincidental error and according to the statistical tests not important in larger studies.

In conclusion we can say that the non-radiochemical HPLC procedure presented has a far better separation capacity for the nucleosides and mononucleotides tested than the radiochemical TLC procedure. Measurements of activities of the purine enzymes 5'NT and PNP by the HPLC procedure are highly reproducible and the time-stability of the HPLC procedure is also better. To guarantee time-stability in the HPLC procedure, 5'NT measurements should be carried out after freezing and storing of the prepared sample, whereas these factors have no influence on the measurement of PNP and HGPRT activity. Since the HPLC procedure is non-radiochemical, it complies better with current environmental protection policies and makes it applicable in common clinical laboratories. Finally this procedure is less time-consuming and more practical when larger numbers of persons have to be screened.

### Acknowledgements

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