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DETERMINATION OF ADENOSINE DEAMINASE AND PURINE NUCLEOSIDE PHOSPHORYLASE ACTIVITIES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND RADIOCHROMATOGRAPHIC METHODS

JIŘÍ BOHÁČEK* and BOHUMIL HOŠEK

Institute of Biophysics, Czechoslovak Academy of Sciences, 612 65 Brno (Czechoslovakia)

and

OLGA BABUŠÍKOVÁ and ANNA HRIVŇÁKOVÁ

Institute of Clinical Oncology, Bratislava (Czechoslovakia)

SUMMARY

Two methods for the determination of adenosine deaminase and purine nucleoside phosphorylase activities were compared. The high-performance liquid chromatographic (HPLC) technique used separation on a reversed-phase silica column and exhibited adequate sensitivity and a markedly higher rate of analysis compared with that of the paper radiochromatographic method. Correlation analysis of the results obtained by the two methods on a set of lymphoid cells from 25 patients with lymphoproliferative disorders confirmed the utility of the HPLC technique in clinical investigations.

INTRODUCTION

The importance of biochemical changes in the purine metabolism of leukaemic cells, not only for the diagnosis of leukaemia subtypes but also for the indication of chemotherapy, has been shown by many workers (for a review, see ref. 1). The published values on adenosine deaminase (EC 3.5.4.4, ADA) and purine nucleoside phosphorylase (EC 2.4.2.1, PNP) activities differ significantly depending on the method of sample processing and the technique used for the determination of the enzymatic activity [2–4].

Methods using radioisotopically labelled substrates with separation of enzymatically formed products by paper chromatography [5], thin-layer chromatography [6] or electrophoresis [4] are considered to be the most reliable reference techniques. Paper radiochromatography (RC) was used in a previous investigation of ADA and PNP activities for the characterization of different forms of

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leukaemia [5]. The determination of purine enzyme activities was recommended as a complementary approach to the leukaemia subtypes classification [5,7].

On the other hand, the technique of radiochromatography is both expensive and time-consuming. Therefore, an intensive search for methods based on other principles was carried out, and high-performance liquid chromatography (HPLC) has also been recommended for the determination of the purine metabolism enzyme activities in human blood cells [8–11]. ADA activity was quantified from the substrate (adenosine) decrease and PNP activity was assessed from the hypoxanthine formed as the enzyme reaction product.

The aim of this work was to determine ADA and PNP activities in lymphoid cells using a previously optimized HPLC method [11] and to compare the results obtained with those given by the paper RC technique [5].

EXPERIMENTAL

Patients and controls

Twenty-five patients with different leukaemias and lymphomas and five healthy controls were studied. Samples of peripheral blood, bone marrow, and lymphatic node were taken before the initiation of any treatment. The diagnosis was made from clinical and haematological symptoms. Four patients had chronic lymphatic leukaemia (CLL) of B phenotype, twelve patients acute myeloid leukaemia (AML) (four with subtype M1–M2 and eight with subtype M4), seven patients non-Hodgkin's lymphoma (NHL) and two patients the T-form of acute lymphoblastic leukaemia (T-ALL).

Isolation and immunological characterization of cells

Mononuclear cells were separated from freshly drawn heparinized peripheral blood or bone marrow on Verografin (SEVAC) as described previously [12]. Immunological phenotype was determined on viable cells in suspension. The binding of well defined monoclonal antibodies and methodical procedures were published [13].

Determination of purine enzyme activities

Radiochromatography. Enzymatic activities were determined after repeated rapid cell freezing ($-70°C$) and thawing. The conditions for enzymatic incubation are given in Table I. Paper RC was followed by the identification of reaction products under UV light, elution of spots corresponding to the appropriate reaction products and their quantification by liquid scintillation counting (Tricarb, Hewlett-Packard, Avondale, PA, U.S.A.). Adenosine (Fluka, Buchs, Switzerland) and [^{14}C]adenosine (Amersham, Amersham, U.K.), specific activity 1.2 GBq/mmol, were the substrates for ADA and inosine (Fluka) and [^{14}C]inosine (Amersham), specific activity 1.5 GBq/mmol, were the substrates for the assessment of PNP activity. Detailed information on paper RC, including data on linearity, was published elsewhere [14].

High-performance liquid chromatography. An HPP 4001 syringe-type pulseless pump with a Model LCD 254 UV detector and CI 100 integrator (Laboratory

TABLE I

DETERMINATION OF ADA AND PNP ACTIVITIES IN LYMPHOCYTES BY RC AND HPLC

Step	RC	HPLC
Substrate for ADA: Ado (4 mmol/l) in Tris buffer (0.1 mol/l); pH = 7.2 (μl)	100 ([^{14}C]Ado)	100
Substrate for PNP: Ino (4 mmol/l) in phosphate buffer (0.1 mol/l); pH = 7.4 (μl)	100 ([^{14}C]Ino)	100
Cell lysate (μl)	100	100
Incubation at 37°C (min)	30	15
Stop reaction (°C; min)	100; 2	100; 5
Centrifugation (min)	10	5
Sample volume (μl)	20	20*
Time of separation (min)	1000	10

*After centrifugation the supernatant was diluted twelve-fold.

Instruments, Prague, Czechoslovakia) was used. Samples were injected using a Rheodyne 7125 syringe-loading valve injector with a 20-μl loop.

The columns used were 75 mm × 3.3 mm I.D. glass columns fixed in a metal cartridge and filled with Separon SIX C_{18} (5 μm) sorbent (Tessek, Prague, Czechoslovakia).

The mobile phases for HPLC were prepared from analytical-reagent-grade potassium dihydrogenphosphate (Lachema, Brno, Czechoslovakia) (20 mmol/l) using triply distilled water and UV-grade methanol (Lachema). Methanol of 10% and 2% (v/v) was used for ADA and PNP determination, respectively. The apparent pH of the mobile phase was 4.4.

ADA and PNP activities were determined in cell lysates prepared by the above procedure. The high selectivity and sensitivity of the HPLC method was exploited for the quantification of nucleosides and bases which are substrates and/or products of the corresponding enzymatic reactions. The conditions for enzymatic incubation and sample preparation are given in Table I.

The statistical significance of the data measured was evaluated using Student's *t*-test and the Wilcoxon matched-pairs signed-rank test.

RESULTS

In the lysates prepared under the same conditions from the same sample of lymphoid cells, the activities of ADA and PNP were determined by means of paper RC and by the fast method using HPLC.

To achieve an adequate selectivity of HPLC separation in cell lysates, two different concentrations of methanol were used in mobile phases for the determination of ADA and PNP activities. Chromatographic data for substances separated by both mobile phases are given in Table II.

The determination of levels of activities was accomplished in groups of patients registered according to the immunological classification of leukaemias. ADA ac-

TABLE II

CHROMATOGRAPHIC DATA FOR SEPARON SIX C_{18} (5 μm)

Glass column, 75 mm × 3.3 mm I.D.; flow-rate, 0.5 ml/min.

Mobile phase		Substance	k'*	N**	Peak asymmetry***
KH_2PO_4 (mmol/l)	Methanol (%)				
20	2	Hyp	2.25	2100	1.2
		Xan	3.04	2200	1.4
		Ino	5.75	3300	1.3
		Ado	19.25	4500	1.5
20	10	Hyp	0.33	1800	1.6
		Xan	0.50	2100	1.5
		Ino	1.00	2800	1.3
		Ado	4.33	3500	1.4

*k' = capacity ratio.
**N = number of theoretical plates (rounded off).
***Peak asymmetry was measured at 10% of the peak height.

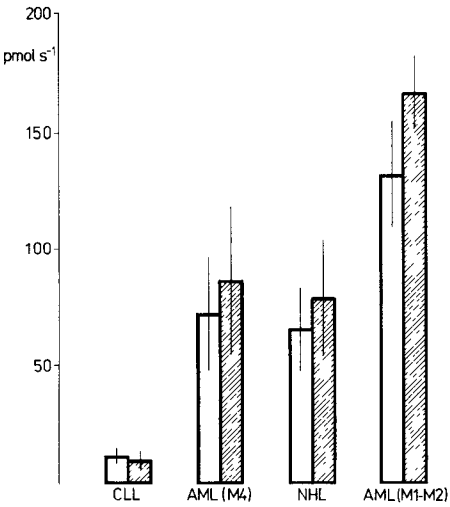

Fig. 1. ADA activity (pmol/s per 10^6 cells) in various groups of leukaemias (for detailed information on individual groups, see Experimental). Open bars, results obtained by HPLC; hatched bars, results measured by RC.

tivity levels determined by both RC and HPLC techniques are presented in Fig. 1. On comparing the results of the two methods, it is obvious that in all but one (CLL) of the groups investigated, HPLC gave lower mean values than those obtained by the RC method, which was considered to be a control method. On average, a difference of about 16% between the results of the two methods was calculated. From the point of view of the individual groups of leukaemias, the activities of ADA in cells from CLL patients were significantly lower than those

in the control group of healthy donors (controls 31.8 ± 7.2 pmol/s per 10^6 cells). The mean ADA activity in CLL patients was one third of the control value. Significantly higher values were observed in the other groups investigated, e.g., NHL and AML. In two cases of the T-ALL (not illustrated) high values of ADA activity (252 and 264 pmol/s per 10^6 cells) were found by the HPLC technique, in agreement with the RC method (257 and 337 pmol/s per 10^6 cells).

In the same groups of patients the PNP activities obtained by HPLC were on average about 20% higher than those given by the RC method (Fig. 2). No statistical differences in PNP activity were found between the various groups of leukaemias, with the exception of CLL patients, where the PNP activity was significantly lower than that in the control group (135 ± 70 pmol/s per 10^6 cells).

Even though differences were found between the mean activities measured by RC and HPLC in the individual groups of leukaemias, these differences were not

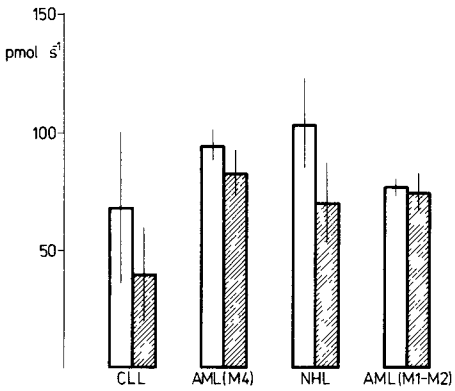

Fig. 2. PNP activity (pmol/s per 10^6 cells) in various groups of leukaemias (for detailed information on individual groups, see Experimental). Open bars, results obtained by HPLC; hatched bars, results measured by RC.

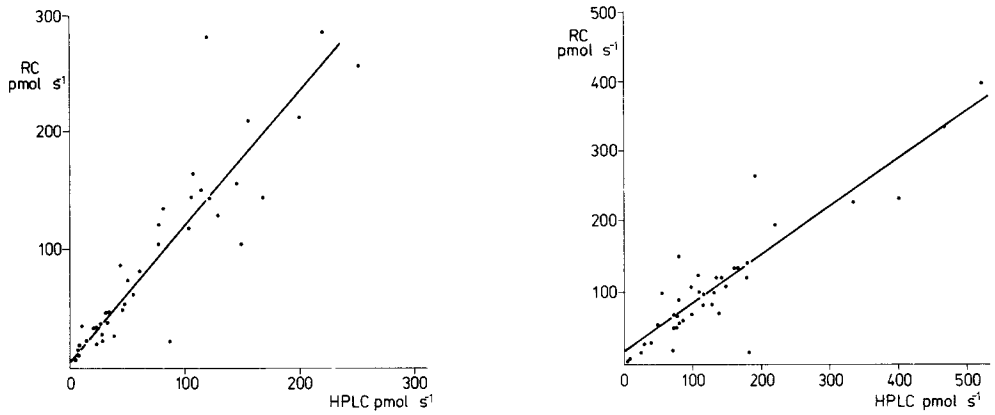

Fig. 3. Graphical relationship of the results for ADA activity (pmol/s per 10^6 cells) obtained by HPLC and RC. Correlation coefficient, $r = 0.918$; regression line, $y = 1.145x + 4.915$.

Fig. 4. Graphical relationship of the results for PNP activity (pmol/s per 10^6 cells) obtained by HPLC and RC. Correlation coefficient, $r = 0.926$; regression line, $y = 0.677x + 10.600$.

statistically significant when evaluated by the Student's t-test. However, when the Wilcoxon's matched-pairs signed-rank test was performed on the whole set of measurements, the hypothesis of coincidence of the results obtained by RC and HPLC must be rejected for both ADA and PNP ($p<0.01$).

A correlation analysis of the activities measured by RC and HPLC for ADA activity is shown in Fig. 3. A correlation coefficient (r) of 0.918 was estimated with high statistical significance ($p<0.001$); the slope of the regression line was 1.1. The analysis of PNP activities measured by both techniques (Fig. 4) exhibited a correlation coefficient of 0.926 ($p<0.001$); the slope of the regression line was 0.68.

To clarify the origin of the systematic differences in the enzyme activities determined by RC and HPLC, measurements were carried out with the lysate of lymphoid cells injected directly on to the chromatographic column after the appropriate dilution with the mobile phase. At 254 nm and at a detector sensitivity of 0.08 a.u.f.s., which are also used in enzyme activity measurements, no detectable peaks were recorded in the region of the retention times corresponding to adenosine, inosine and hypoxanthine.

DISCUSSION

Currently available methods for ADA and PNP activity determination exploit various chemical, physical and/or radiochemical principles [2]. The high separation efficiency and sensitivity of HPLC permits the use of non-isotopically labelled substrates for the determination of purine enzyme activity. The speed of the analytical HPLC procedure is two orders of magnitude higher than that of the reference paper RC approach (see Table I). The results of enzyme activity measurements obtained on an identical set of lymphoid cell lysates enable us to compare statistically the two analytical approaches. The samples analysed were taken from leukaemic patients to verify the practical usefulness of the significantly faster HPLC technique.

ADA activities using the RC method were calculated from the sum of radioisotopically labelled inosine and hypoxanthine. For the determination of ADA using HPLC a simplified approach to evaluation was selected. A decrease in the substrate adenosine concentration after 15 min of enzymatic conversion was employed to evaluate the ADA activity level [9]. The results obtained using HPLC agree well with those given by RC for ADA activity levels below 100 pmol/s per 10^6 cells. Above this level a systematic lowering of ADA activity took place, on average 20%. Dilution of high-activity samples may be used to overcome this effect.

Using the Wilcoxon's matched-pairs signed-rank test, coincidence of the results over the full activity range was not proved, but correlation analysis confirmed a significant relationship between ADA and/or PNP activity levels determined using HPLC and RC. PNP activities were calculated from the formation of the product of the enzymatic reaction, i.e., hypoxanthine in HPLC and [^{14}C]hypoxanthine in RC. The systematic elevation of PNP activities measured by HPLC may partly be explained by the above-mentioned different principles

used for calibration in the two procedures. The main source of the methodical errors in both procedures was the cell lysate preparation, including cell counting. Hence the precision of the HPLC technique could not be fully exploited. Carrying out repeated calibrations with suitable standards of nucleosides or bases is a prerequisite for obtaining reproducible data.

With the exception of CLL, where the PNP activities were lowered, the other leukaemia subgroups investigated exhibited only non-significant changes in comparison with a control group of healthy individuals. Therefore, some workers have used only the determination of ADA activity levels to differentiate between leukaemia subtypes [15].

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