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Note

Rapid determination of purine enzyme activity in intact and lysed cells using high-performance liquid chromatography with and without radiolabelled substrates

L.D. FAIRBANKS*, A. GODAY, G.S. MORRIS, M.F.J. BROLSMA, H.A. SIMMONDS and T. GIBSON

Purine Laboratory, Clinical Science Laboratories, Guy's Hospital Medical School, London Bridge, SE1 9RT (Great Britain)

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Rylance et al. [1] recently published methods enabling rapid screening for inherited enzyme defects of purine metabolism associated with uric acid overproduction, using high-performance liquid chromatography (HPLC). The advantage of such methods is their speed as well as the cost reduction and the avoidance of the use of radiolabelled substrates. The drawback is that they may only be used to measure enzyme activity directly in lysed cell preparations employing a specific substrate for each individual enzyme.

This paper describes an adaption of the method of Rylance et al. [1] to a rapid fully automated HPLC system which can be used with an on-line radiodetector for intact cell studies. The use of radiolabelled substrates is essential for the latter in order to evaluate the metabolic fate of a particular substrate. The method described for the simultaneous monitoring of UV absorbance and radioactivity is more rapid than those recently published [2].

METHODS

Studies in intact and lysed cells

The methods used for the enzyme assays in cell lysates, as well as metabolic studies in intact cells, have been described in previous publications [3, 4]. The enzyme assays investigated by HPLC included adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), adenine (APRT) and hypoxanthine—guanine phosphoribosyltransferase (HGPRT) and phosphoribosyl-pyrophosphate synthetase (PP-rib-PS).

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All incubations using lysed cells were carried out for 15 min at 37°C, initially using radiolabelled substrates (8-^{14}C-labelled adenosine, inosine, adenine and hypoxanthine and/or guanine respectively). The assays were terminated either by the addition of 40% trichloroacetic acid (TCA) followed by extraction to neutrality with water-saturated diethyl ether (APRT, HGPRT), or heating at 100°C for 2 min, (ADA, PNP) [3, 4].

Extracts were run in duplicate on the HPLC system described below, using a dual-channel detector capable of simultaneous monitoring of absorbance at 254 nm and 280 nm. Duplicate extracts were then re-run on the high-performance liquid chromatograph, using a radiodetector in place of the 280-nm UV monitor, allowing simultaneous comparison of the distribution of the label in substrate and product(s) with the 254-nm UV trace.

For the intact cell studies, $1 \cdot 10^6$ cells were incubated for 2 h at 37°C with 25 μM [8-^{14}C]deoxyadenosine (New England Nuclear) in RPMI (Flow Laboratories) supplemented with 10% heat-inactivated foetal calf serum (FCS). The final volume of the incubation mixture was 100 μl (specific activity 37 kBq/ml).

Cells and medium were separated by centrifugation (400 g, 5 min at 4°C) and the pellet washed once (400 g, 5 min at 4°C) with 200 μl of ice-cold 0.85% sodium chloride.

Medium and pellet were mixed with cold TCA (25 μl of 40% TCA and 100 μl of 8% TCA, respectively) and centrifuged for 1 min (12,000 g, Beckman Microfuge). Supernatants were extracted to neutrality with water-saturated diethyl ether and analysed by HPLC as for the red cell lysates.

Chromatography

The HPLC system used was a Waters Assoc. (Cheshire, Great Britain) tri-module fully-automated system, consisting of a WISP 710B automatic injector, a twin pump Model 6000A solvent delivery system, a Model 440 dual-channel UV detector (254 nm, 280 nm), linked to a System Controller and Data Module integration and printout system. The radiodetector was a Precision Radioactivity Monitor with a heterogeneous flow cell of 200 μl capacity supplied by Reeve Analytical (Glasgow, Great Britain).

A Z-module radial compression system, containing a 10-μm reversed-phase radial-Pak C_{18} cartridge (100 × 8 mm I.D.), was used for the lysed cell separations. Buffer A contained KH_2PO_4 2.7 g/l (20 mM) pH 4.45; Buffer B consisted of methanol—water (60:40). A linear gradient (gradient 6) was used for all separations.

A gradient increasing to 40% B in 17 min was used for the ADA assay, with a 3-min equilibrium delay between injections. For the PNP assay, gradient 6 to 30% B in 10 min was used. For APRT and HGPRT, the same gradient to 32% B and 24% B, in 9 min and 8 min, respectively, was used. The flow-rate was 3 ml/min in all instances.

For the intact cell studies, the 280-nm channel recorder was disconnected and a Reeve Analytical radioactivity monitor was attached to the Data Module of the trimodular system (pen 1) for continuous monitoring of radioactivity, in parallel with the UV absorbance at 254 nm using pen 2. The radiodetector contained a flow cell (200 μl void volume) packed with solid scintillant (99/

3811 GSI glass scintillant powder, Grade W, 63—80 μm) obtained from Koch-Light (Colnbrook, Great Britain).

For detection of radioactivity in nucleosides and bases in the medium, the above reversed-phase system was employed. For the nucleotides in the cell extracts, a Partisil 10 SAX cartridge (10 μm, 100 × 8 mm I.D.) was used. This anion-exchange system employed a phosphate gradient: Buffer A contained KH_2PO_4 0.68 g/l (5 m*M*) pH 2.65; Buffer B 25 g/l KH_2PO_4 plus 25 g/l KCl, pH 3.85. The flow-rate was 2 ml/min using a linear gradient (gradient 6) increasing to 100% B in 20 min.

The phosphate used was Aristar grade from BDH Chemicals (Poole, Great Britain), the potassium chloride Analar grade also from BDH. The methanol used was a special HPLC grade from Rathburn Chemicals (Walkerburn, Great Britain).

RESULTS

Cell lysates

A typical chromatogram for each of the four different enzyme assays obtained by HPLC is given in Fig. 1a—d. Fig. 1e shows the radiodetector trace

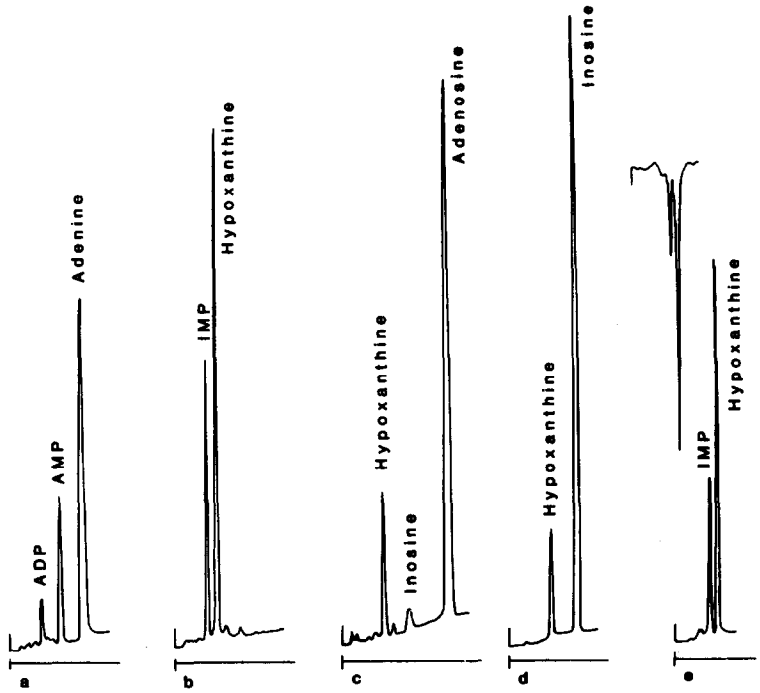

Fig. 1. Representative HPLC trace at 254 nm obtained for four different enzyme assays using haemolysate of healthy controls. (a) 10-μl injection of extract from a typical APRT assay; (b) 10-μl injection of extract from an HGPRT assay; (c) 50-μl injection of assay extract from an ADA assay; (d) 5-μl injection of assay extract from a PNP assay; (e) 7-μl injection of assay for HGPRT as shown in (b). The HPLC trace at 245 nm (lower) printout is compared (in e) with the radiodetector (upper) printout in place of the 280-nm channel printout. The traces were recorded at 0.1—0.5 absorbance units full scale (a.u.f.s.) and a chart speed of 0.2 cm/min. Other conditions are given in detail in the Methods section.

(upper) from an HGPRT assay compared with the direct HPLC assay (lower) demonstrating that these enzymes can be assayed rapidly in lysed cells using labelled or unlabelled substrate. Furthermore, they confirm the reproducibility of the method. Quadruplicate analyses agreed within 2%. Day-to-day variability was also within this range but varied in the long term with the age of the column which was controlled by external standard quantification.

Difficulties were encountered in the use of HPLC alone for the PP-rib-PS assay because the first step utilises ATP which is converted to AMP in the process [5]. Since AMP is also a product of the second step, the enzyme activity could thus not be determined directly by monitoring the UV absorption by HPLC of substrate and product. The assay could be applied to HPLC by using [8-^{14}C]adenine in the second step and using the radiodetector as in the intact cell studies to evaluate the percentage conversion of substrate.

Intact cells

Fig. 2 shows the metabolism of deoxyadenosine (dAR) using a normal lymphocyte cell line [6] established in long term culture using Epstein Barr

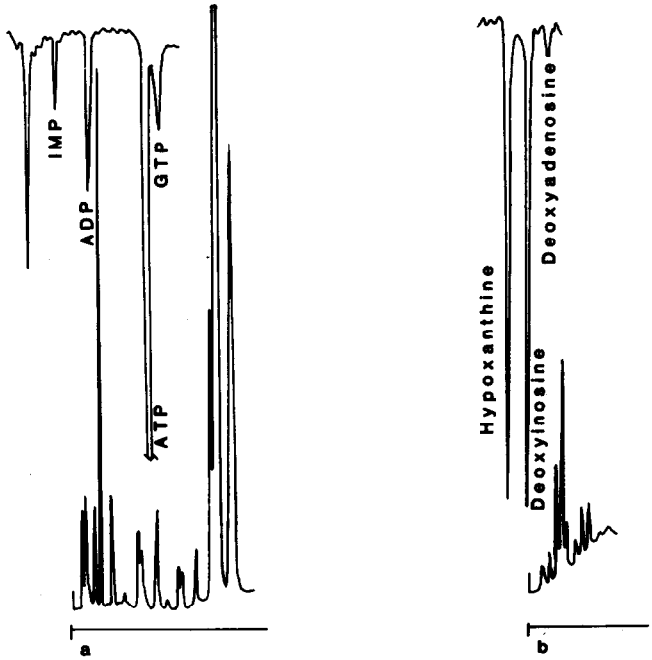

Fig. 2. HPLC trace showing the metabolism of deoxyadenosine in an Epstein Barr virus genome positive B cell line (Wil) established from normal lymphoblasts [6]. (a) Trace obtained from the cell extracts by simultaneous monitoring of UV absorbance and radioactivity, using the anion-exchange system described in the Methods section, showing the incorporation of radioactivity into the different nucleotide pools: IMP, ADP, ATP, GTP. The peak eluting at the front of the chromatogram corresponds to radioactivity in the medium not removed by centrifugation. 0.05 a.u.f.s., chart speed 0.1 cm/min. (b) The HPLC trace of the medium, after incubation, using the reversed-phase system described in methods. The UV trace at 254 nm is recorded in the (lower) printout at 0.05 a.u.f.s., chart speed 0.15 cm/min. The radiodetector trace is recorded in the (upper) printout, in place of the normal HPLC trace from the 280-nm UV detector.

(EB) virus. Fig. 2b shows a typical radiolabel trace (upper) obtained following a 25-μl injection of medium, showing that distribution of radiolabel is predominantly in deoxyinosine (dHR) and hypoxanthine, due to the rapid degradation of dAR by ADA and PNP. Within the cell (Fig. 2a), further metabolism of hypoxanthine to IMP by HGPRT has resulted in counts being predominantly in the peaks corresponding to ATP and ADP. Some of the substrate has also been metabolised to [8-^{14}C]GTP as indicated in the upper trace obtained with the injection of 75 μl of cell extract (Fig. 2b).

DISCUSSION

The use of a fully automated system for the type of studies described here is advantageous for several reasons. First, the speed with which a large number of samples can be processed; both the APRT and HGPRT assays can be run at a rate of six per hour, the PNP and ADA assays at approximately three per hour. Secondly, the 48-sample capacity of the fully automated system allows overnight analysis. Furthermore, the results obtained by HPLC agree closely in all instances with the values obtained previously where reaction products were separated first by high-voltage electrophoresis on thin-layer plates, visualised under UV, scraped directly into scintillation vials and subsequently counted. The saving in cost as well as time is also obvious — one step instead of four.

The ability to couple the radiodetector to the high-performance liquid chromatograph has proved invaluable for the intact cell studies. Particularly for the nucleotides, where it was not always easy in the past to separate all the different nucleotides using high-voltage electrophoresis. The use of the Z-module enables rapid change from the reversed-phase system to the anion-exchange system, and is invaluable for the intact cell studies.

We have investigated the application of a fifth enzyme assay, PP-rib-PS to HPLC analysis. However, in our hands this did not prove satisfactory because the assay is a two-step process using ATP in the first step to generate PP-rib-P. However, using [8-^{14}C]adenine in the second step together with the radiodetector attachment, as for the intact cell studies, the synthetase assay could be processed by HPLC.

These studies confirm that not only can much of the tedium and cost of routine enzyme assays be overcome by adaption to HPLC, but the system can also be used as a valuable tool for research purposes.

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