Journal of Chromatography, 415 (1987) 383–387
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3529

Note

Sensitive on-line radioactivity measurements with a heterogeneous flow-cell: application to lymphoid cell ribonucleotides separated by high-performance liquid chromatography

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(Received October 6th, 1986)

Incubation of biological material with labelled metabolic precursors, followed by chromatographic separation of the various metabolites and measurement of their radioactivity, has proved its value for the study of biochemical pathways. However, the quantitation of low amounts of radioactivity in the metabolites remains difficult. The separation and collection of fractions followed by radioactivity quantitation with liquid scintillation is an expensive and time-consuming procedure. The fractions have to be small, in order to ensure discrete counting of the separated metabolites. Moreover, the samples are lost for further analysis.

Another possibility is the use of on-line radioactivity detection, but until now this method has shown low sensitivity [1], owing to the lack of adequate scintillators for dynamic measurements and to the inadequate construction of the detection system. The sensitivity can be enhanced by the use of homogeneous systems, in which the eluate from the column is mixed with scintillation fluid before entering the flow-cell of the radioactivity detector [2].

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In the present paper, we describe an on-line radioactivity detector with a heterogeneous flow-cell (containing scintillation crystals). This system operates with appreciably lower costs than fraction collection followed by liquid scintillation counting. It has a high dynamic counting efficiency in combination with a low background, without loss of chromatographic resolution. Moreover, the eluate is still available for further analysis. As an application of this system the measurement of the incorporation of [^{14}C]uridine into pyrimidine metabolites by lymphoid cells is described.

EXPERIMENTAL

Chemicals

The nucleotides used as chromatographic standards and Sigmacoat® were obtained from Sigma (St. Louis, MO, U.S.A.). [U-^{14}C]uridine (19.57 GBq/mmol) and [8-^{14}C]adenosine (2.04 GBq/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.). Tridistilled water was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Fetal calf serum was purchased from Flow Labs. (Irvine, U.K.) and RPMI 1640 medium with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) from Gibco (Paisley, U.K.). Mucasol® was obtained from Merz (Frankfurt, F.R.G.) and all other chemicals used were of analytical grade and obtained from Merck (Darmstadt, F.R.G.).

Incubation of cells

Cells from the human lymphoblastic cell line MOLT-3 [3] were maintained in humidified air–5% carbon dioxide at 37°C in RPMI 1640 medium with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal calf serum. Cells were kept in exponential growth by dilution to $5 \cdot 10^5$ cells/ml every two to three days. Normal blood lymphocytes were isolated as previously described [4] and cultured for 18–20 h under the same conditions. The incubation with [^{14}C]uridine (37 kBq/ml cell suspension) was performed for 2 h.

High-performance liquid chromatographic (HPLC) analysis of ribonucleotides

Nucleotides were extracted with 0.4 M perchloric acid and separated with an anion-exchange HPLC method, as described elsewhere [5]. Columns were pre-packed Partisil-10 SAX cartridges (10×0.8 cm I.D.) radially compressed in an RCM-Z module (Waters Assoc., Milford, MA, U.S.A.). The separations were performed at a flow-rate of 2 ml/min.

On-line radioactivity measurement

The radioactivity incorporated in the various ribonucleotides was monitored by means of an on-line heterogeneous system, the Ramona D (Isomess, Straubenhardt, F.R.G.) in combination with an Apple IIe computer for quantification of the digital signal with dual-trace Radio-Chromato-Graphic System software (IM 2006; Isomess).

A flow-cell filled with modified yttrium silicate (X) or calcium fluoride (CaF_2) as scintillation crystals was used (Isomess). Both flow-cells had a volume of 600 μl and were internally siliconized with Sigmacoat.

RESULTS

Efficiency measurements

The efficiencies of the two different scintillators (CaF_2 and X) were determined by repeated (three times) injection of a [^{14}C]adenosine solution with known activity, as determined by liquid scintillation counting. The efficiency of the liquid scintillation counter was determined by the standard addition method. The dynamic counting efficiency of the flow-cell is the ratio of the counts per minute measured after injection into the HPLC system (without column) and the disintegrations per minute of the adenosine solution. For the elution of the various ribonucleotides from the anion-exchange column, a salt and pH gradient is used [5]. The high salt concentration at the end of this gradient caused no quenching of the activity.

The dynamic counting efficiency of the CaF_2 cell was 28%, and that of the X cell 42.8%, measured with a lower energy threshold of 75 keV. The efficiency of ca. 43% was also found for the nucleotide components in labelled cell extracts (see *HPLC analysis of ribonucleotides*). When the whole energy spectrum from 0 to 500 keV was measured, the efficiency was twice as high, but at the same time the background and its standard deviation increased at least ten times. To ascertain a good sensitivity, further experiments were performed with a lower threshold of 75 keV.

Because of the higher dynamic counting efficiency we used the X cell for our experiments.

Background

When used as supplied, the backgrounds of the X and CaF_2 flow-cells increased rapidly by adsorption of labelled compounds. The flow-cells could be cleaned by means of repeated injections with alkaline soap (10% solution of Mucasol) after removal of the HPLC column. However, the adsorption of label on the scintillation crystals could be prevented by internal coating of the flow-cells with Sigmacoat. The background activity of coated cells was 0.2 ± 0.1 cps (mean $\pm$ S.D.; window 75–500 keV) and increased only slowly in time. When the background was above 1 cps, the flow-cell was cleaned.

Linearity and detection limit

To control the linearity of the detection system and to determine the detection limit, various amounts (1–100 μl) of two [^{14}C]adenosine solutions (3.7 and 37 Bq/μl, respectively) were injected into the HPLC system with the anion-exchange column and the radioactivity was quantified by integration of the digital signal from the Ramona D. A linear correlation was found between the injected and the measured activity. At a flow-rate of 2 ml/min, peaks of 5 Bq were three times the standard deviation above the background radiation. Therefore, the detection limit

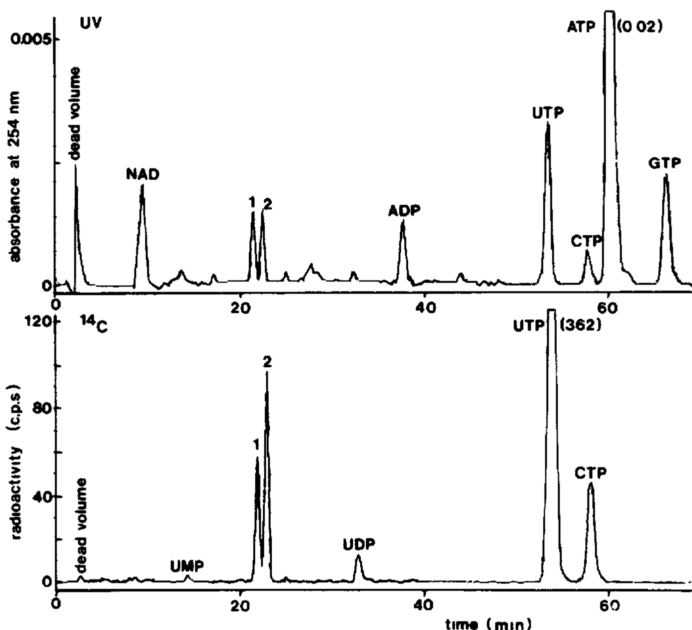

Fig. 1. HPLC-UV and HPLC-radioactivity (^{14}C) profile of a perchloric acid extract of MOLT-3 cells after incubation with [^{14}C]uridine for 2 h as described under Experimental. Sample equivalent to 10^6 cells and 3.2 kBq. Peaks: 1 = UDP-N-acetylglucosamine and/or UDP-N-acetylgalactosamine; 2 = UDP-glucose and/or UDP-galactose. c.p.s. = counts per second.

was taken as 5 Bq. When the analogue signal of the Ramona D was integrated with a computing integrator 308 (Infotronics Laboratory Data Control, Shannon, Ireland), the minimal radioactivity that could be quantified was ca. 35 Bq at a count range from 0 to 400 cps.

Analysis of the ribonucleotides

Fig. 1 shows the results of HPLC analysis and on-line radioactivity detection of ribonucleotides in lymphoblasts of the MOLT-3 cell line after 2 h of incubation with [^{14}C]uridine. Compared with the UV profile, the radioactivity profile showed labelling of some specific peaks only. The separation between the various peaks was similar in both profiles. Rechromatography of a particular peak resulted in one peak, both in the UV and radioactivity profile (not shown), indicating that no mixing occurred in the flow-cell of the radioactivity monitor. As might be expected, most of the label from [^{14}C]uridine was incorporated in the uracil nucleotides, especially UTP and the UDP sugars. A relatively large amount (10%) of the label was recovered in CTP. The latter phenomenon is dependent on the cell type: in normal lymphocytes the incorporation of uridine in the cytosine nucleotides was much lower (Table I).

DISCUSSION

Modified yttrium silicate (X), a new scintillator for heterogeneous on-line radioactivity measurements, was shown to have 1.5 times the dynamic counting

TABLE I

DISTRIBUTION OF [^{14}C]URIDINE OVER THE VARIOUS PYRIMIDINE RIBONUCLEOTIDES IN NORMAL BLOOD LYMPHOCYTES AND MOLT-3 CELLS AFTER 2 h OF INCUBATION

Values are expressed as the percentage of the total incorporated label, and are the means of three experiments, with a coefficient of variation of maximal 30%.

Pyrimidine ribonucleotide	Normal blood lymphocytes (8.1 pmol per 10^6 cells incorporated in extract)	MOLT-3 cells (165.6 pmol per 10^6 cells incorporated in extract)
Uracil nucleotides	69.6	69.5
Cytosine nucleotides	4.3	10.3
UDP sugars	25.5	20.2
Cytidine diphospodiesters	0.9	N.D.*

*N.D. = not detectable.

efficiency of the frequently used CaF_2. A flow-cell with the scintillator X in a new type of radioactivity detector, the Ramona D, in combination with integration of the digital signal, was shown to be very sensitive.

On-line heterogeneous radioactivity measurement in HPLC eluates is able to compete with fraction collection followed by liquid scintillation counting, because the flow-cell system does not suffer from loss of performance during the chromatographic analysis, and is less expensive and time-consuming than fraction collection followed by liquid scintillation counting. Also the amount of radioactive and/or organic waste is much less. Although homogeneous flow-cell systems are more sensitive than the heterogeneous types [2], the latter have the advantage that the sample is not lost by mixing with a scintillation fluid. Thus, the eluate is available for further analysis, e.g. repeated chromatography, thin-layer chromatography or special identification reactions.

The results of the incubation experiments showed that HPLC in combination with heterogeneous radioactivity detection is a very useful technique to determine radioactive metabolites. Investigations of cell metabolism by comparison of the results after incubation with radioactive precursors under various conditions for the same cell type or under the same conditions for different cell types are possible, and even small differences in incorporation patterns can be determined.

ACKNOWLEDGEMENT

This study was partly supported by the Queen Wilhelmina Fund/Netherlands Cancer Organization (Grant No. AUKC-EMMA 81-5).

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