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APPLICATION OF SIMULTANEOUS UV–RADIOACTIVITY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE STUDY OF INTERMEDIARY METABOLISM

I. PURINE NUCLEOTIDES, NUCLEOSIDES AND BASES

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SUMMARY

Procedures are presented for the continuous-flow, simultaneous measurement of concentration and radioactivity of purine metabolites separated by high-performance liquid chromatography (HPLC). A microprocessor-controlled radioactivity flow detector connected in series to a UV-flow detector provides on-line quantitative monitoring of separated components in the post-column effluent stream. Through use of two HPLC separations — reversed-phase and anion-exchange — quantitation of all major purine nucleotides, nucleosides and bases is possible. The procedures provide a rapid, sensitive, and convenient means for the systematic study of purine metabolism.

INTRODUCTION

Purine metabolites and their intermediary metabolic pathways are of major importance in the study and development of antiparasitic, antimicrobial and antineoplastic agents. In addition, the recognition of purine enzyme defects associated with immunodeficiency diseases has emphasized the relevance of understanding biochemical abnormalities of purine metabolism in these diseases[1].

Numerous investigators have exploited the advantages of high-performance liquid chromatography (HPLC) for the quantitative analysis of purine bases, nucleosides and nucleotides[2–10]. Methods currently in use employ a variety of column packing media and mobile phase compositions. Depending on the choice of column, eluent conditions and detector, it is possible to obtain sensitive and reproducible profiles for most purine compounds.

Although information on the concentrations of purine metabolites in a sample preparation can be quite valuable, this information alone does not permit detection of changes in metabolic pathways. Consider, for example, that a defect in one pathway of purine metabolism may be associated with compensatory changes in another pathway such that quantitative changes in purine metabolites are not detected. The dy-

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namic aspects of a metabolic pathway may, however, be studied by use of radioisotopes. By addition of isotopically labelled compounds to a metabolic system, it is possible to gain information on the distribution of radiolabel within pathway intermediates and to develop data on their metabolic fate, as well as their rates of production or interconversion. Such requirements can be presently met by HPLC separations in which the column effluent is collected in fractions and analyzed by conventional liquid scintillation counting techniques and equipment. Although instrumentation for continuous counting of aqueous solutions containing weak β-emitters (^{14}C and ^{3}H) with liquid scintillators has been developed, these systems have been experimental in design and built for adaptation to large-scale conventional liquid scintillation spectrometers[11,12].

We report here on a novel and informative approach to the study of purine metabolism that involves coupling of a commercially available radioactivity detector on-line with a HPLC system and standard UV-detector. Depending on the type of separation (anion-exchange or reversed-phase), the system measures simultaneously the concentration and radioactivity for a given chromatographic peak. Rapid and reproducible results may be obtained and recorded as both analog (peak trace) and digital output (peak integration). Through the use of various radiolabelled purine precursors, it is possible to study in a systematic way the overall metabolism of purine nucleotides, nucleosides and bases.

EXPERIMENTAL

Apparatus

Chromatographic determinations were done with a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 3500B liquid chromatography system: two 740B pumps, a 744 solvent programmer, a 755 sample injector (123 μl loop) and a System I computing integrator. UV absorbance was measured using an Altex (Berkeley, CA, U.S.A.) Model 152 dual-wavelength (254 and 280 nm) detector with a 20-μl analytical flow cell.

Radioactivity was measured using a Flo-One (Radiomatic Instruments, Addison, IL, U.S.A.) radioactive flow detector with a 500-μl flow cell. The unit had two photomultiplier tubes, a variable volume, valveless piston pump for delivery of scintillator fluid and was microprocessor controlled. Integrator programs built into the microprocessor compute output in radioactive units as counts per unit time or as counts per peak and total counts per sample. The unit was designed for detection of low energy, β-emitting radionuclides with two operating channels permitting operation of the unit in a double-labelled mode. The radioactivity detector was connected on-line to the HPLC system in series with the UV detector so that column effluent first passed through the UV-detector, then through the radioactivity detector where it was mixed with scintillator fluid (pre-determined ratio) before passing into the flow cell and out to waste or collected. Mini-Scint (Radiomatic Instruments) liquid scintillator was used for all analyses.

HPLC separation of nucleosides and bases

Purine nucleosides and bases were separated by reversed-phase on an Ultrasphere-5 ODS (Altex) high-density packed microparticulate column (250 × 4.6 mm

I.D.). The mobile-phase composition was: low (A) eluent 0.01 *M* KH_2PO_4 (pH 5.6); high (B) eluent methanol–water (70:30)[7]. The low eluent was pumped (flow-rate, 1 ml/min) isocratically for 3 min followed by a linear gradient (0–40% B in A over 30 min). Concentration was determined by an external standard method based on absorbance (254 nm) using purine standards of known purity.

HPLC separation of nucleotides

Purine nucleotides were separated on a Partisil-10 SAX (Whatman, Clifton, NJ, U.S.A.) strong anion-exchange microparticulate column (250 × 4.6 mm I.D.). Mobile phase composition was as follows: low (A) eluent 0.01 *M* KH_2PO_4 (pH 3.4); high (B) eluent, 0.8 *M* KH_2PO_4 (pH 4.3). The low eluent was pumped isocratically for 11 min after which a linear gradient was generated (0–80% B in A over 30 min). The flow-rate was 1.6 ml/min. It was necessary to recrystallize[13] the potassium phosphate to remove a UV impurity[4] which caused a severe rise in baseline at detector settings of 0.04 absorbance units full scale (a.u.f.s.) or lower. An external standard method was used for quantitation of concentrations based on absorbance (254 nm).

Chemicals and isotopes

Potassium phosphate was obtained as the NF grade and recrystallized (Mallinckrodt, St. Louis, MO, U.S.A.). Purine nucleotides, nucleosides and bases were of the highest quality and purity (Sigma, St. Louis, MO, U.S.A.). Methanol was HPLC grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). All isotopes were purchased from New England Nuclear (Boston, MA, U.S.A.) or Amersham (Arlington Heights, IL, U.S.A.). Each isotope was analyzed for purity using the appropriate HPLC separation method. The isotopes and specific activities were: [^{14}C]AMP (48.8 mCi/mmole), [8-^{14}C]ADP (44.8 mCi/mmole), [8-^{14}C]ATP (56.4 mCi/mmole), [8-^{14}C]hypoxanthine (52.8 mCi/mmole), [8-^{14}C]deoxyadenosine (536 mCi/mmole), [8-^{14}C]adenine (56.2 mCi/mmole), [8-^{14}C]adenosine (58 mCi/mmole), and [2,8-^{3}H]adenine (16 Ci/mmole).

RESULTS

HPLC quantitation of purine compounds

Table I gives the retention times and areas for purine standards separated by the described methods. The separations were highly reproducible so long as operating parameters and mobile phase compositions were kept constant. Fig. 1 shows the reversed-phase separation of a mixture of eleven purine nucleoside and base standards of known purity to which four isotopically labelled [^{14}C]purines were added. This figure is a tracing of the strip-chart recorder output (channel A is UV (254 nm) on a linear scale and channel B is radioactivity traced as logarithmic output). The radioactive peak follows directly behind the UV peak by a time factor that is proportional to the rate of flow between detector flow cells. The separation of adenosine from deoxyadenosine typifies the resolution obtained for closely related peaks. Similar results were obtained for purine nucleotides separated by anion-exchange gradient chromatography.

Determination of radioactivity flow cell efficiency

In a radioactive flow detector, even though the counting efficiency of the cell in

TABLE I

RETENTION TIMES AND AREAS FOR PURINE BASES, NUCLEOSIDES AND NUCLEOTIDES

Standard error of mean (S.E.M.) for retention time values is calculated from five runs. Values for UV area represent integrated areas for peaks computed by data system (counts ± S.E.M., $n = 5$).

Compounds	*Retention time (sec ± S.E.M.)*	*UV area (Area ± S.E.M.)*	*Compounds*	*Retention time (sec ± S.E.M.)*	*UV area (Area ± S.E.M.)*
Uric acid	390 ± 2	4140 ± 38	Adenosine monophosphate (AMP)	474 ± 3	8948 ± 31
Hypoxanthine	681 ± 3	15,049 ± 85	Inosine monophosphate (IMP)	986 ± 5	4354 ± 37
Xanthine	805 ± 3	4781 ± 38	Guanosine monophosphate (GMP)	1193 ± 6	8363 ± 53
Xanthosine	1116 ± 3	4707 ± 32	Xanthosine monophosphate (XMP)	1291 ± 2	7585 ± 49
Adenine	1419 ± 3	14,571 ± 131	Adenosine diphosphate (ADP)	1726 ± 2	6897 ± 15
Inosine	1504 ± 3	9333 ± 24	Guanosine diphosphate (GDP)	1876 ± 2	6839 ± 23
Guanosine	1562 ± 3	6825 ± 39	Adenosine triphosphate (ATP)	2497 ± 2	4728 ± 29
Deoxyinosine	1603 ± 4	8299 ± 51	Guanosine triphosphate (GTP)	2731 ± 2	3626 ± 38
Deoxyguanosine	1677 ± 3	8193 ± 66			
Adenosine	2030 ± 4	8437 ± 152			
Deoxyadenosine	2118 ± 4	8285 ± 157			

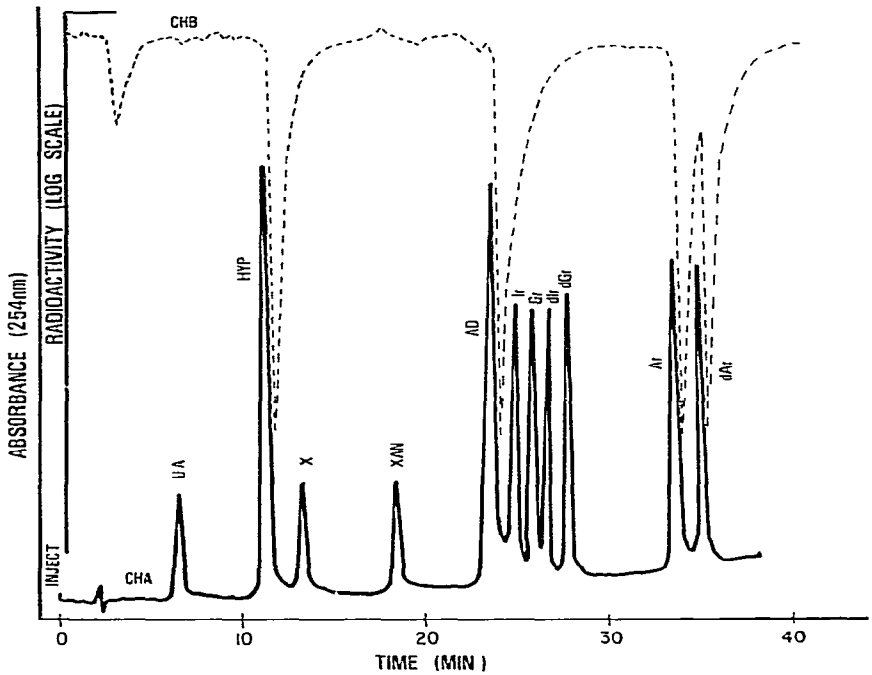

Fig. 1. Simultaneous UV–radioactivity reversed-phase HPLC separation of nucleosides and bases. Injection volume: 123 μl of a standard solution containing 2–3 · 10^{-6} g, of each compound. Radioactivity added was 0.5–1 · 10^{-3} μCi/μl of each compound based on commercial specifications. Column: Ultrasphere ODS (5 μm particle size); eluents: low strength (A), 0.01 *M* KH_2PO_4, pH 5.6; high strength (B), 70% methanol in water; gradient 0–40% B in A over 40 min, linear; temperature: ambient; flow-rate: 1.0 ml/min. UV detector was set at 0.04 a.u.f.s. —scale was linear. Radioactivity detector output was logarithmic— full recorder range was divided into four logarithmic decades with full scale = 1 · 10^6 cpm. Peak identity: U.A. = uric acid; HYP = hypoxanthine; X = xanthine; XAN = xanthosine; AD = adenine; Ir = Inosine; Gr = guanosine; dIr = deoxyinosine; dGr = deoxyguanosine; Ar = adenosine; dAr = deoxyadenosine. CHA, channel A (254 nm); CHB, radioactivity.

a "static mode" is the same as for conventional liquid scintillation counting, the expected count rate in the dynamic (flow) mode will not be the same. To assess counting efficiency in the dynamic mode, the expected count rate (ECR) was calculated as follows:

$$\mathrm{ECR} = \mathrm{dpm_s} \cdot \frac{\mathrm{SE}\,(\%)}{100} \cdot \frac{V}{F}$$

Where ECR is the number of total counts expected from a given sample during the sample residence time. The residence time is that interval of time during which a segment of sample is counted by the photomultiplier tubes. It is determined by the flow cell size and the rate of flow through the flow cell (V/F). $\mathrm{dpm_s}$ are disintegrations per minute in the given sample. SE (%) is the true counting efficiency of the flow cell with the given sample mixture in the static mode (*i.e.* held stationary in the flow cell for counting over a set time period); the SE is calculated as cpm × 100/(dpm/ml × V). V is the active volume (as seen by the photomultiplier tubes) of the cell in milliliters

(0.5 ml in the Flo-One). F is the total flow-rate through the cell at the detector outlet in ml/min.

The ECR can be evaluated in relation to the chromatographic system's specific operating conditions by calculation of the apparent efficiency. As used here, apparent efficiency is defined as a measure of the radioactivity detector's dynamic counting efficiency for a sample during its residence time in the flow cell. The apparent efficiency is calculated from the ratio of observed counts to expected counts divided by the reciprocal residence time. When the observed count rate is equal to the ECR, a maximum value is obtained for the apparent efficiency under the specific operating conditions. Table II shows the ECR and apparent efficiencies obtained for the reversed-phase separation of purine nucleosides and bases. The observed counts were in good agreement with the computed ECR, there being less than a 6 ± 0.9% difference in the ratio (observed counts:ECR) for all cases. This ratio expressed as a percent represents the "recovery" of radioactive counts as detected under the system operating conditions when compared to the actual radioactivity present. As such, the "recovery" value represents an estimate of the systems' reproducibility.

In that the reversed-phase separation involves a gradient, the change in composition of the mobile phase (ratio of eluent B to eluent A or % B in A) could affect counting efficiency as a result of changing chemical quench. To evaluate the presence of gradient produced quench, a series of discrete static efficiencies were determined by reconstructing the gradient in 5% increments. Over the reversed-phase gradient, the static efficiency was not observed to change significantly from that recorded for isocratic (0% B in A) conditions. The observed static efficiency was 91%.

Table III gives the ECR and apparent efficiencies observed for the anion-exchange separation of purine nucleotides. The overall "recovery" of radioactive counts (observed counts:ECR) was 94 ± 1.7% for all three nucleotides measured (standard solution and dilutions). The observed apparent efficiencies compared well

TABLE II

APPARENT EFFICIENCIES FOR PURINE NUCLEOSIDES AND BASES

Apparent efficiency calculated as described in text. Standard solution containing both radioactive and non-radioactive purines of known purity.

	Standard solution				*1:2 Dilution*		*1:4 Dilution*	
	dpm$_s$*	*ECR*	*Observed counts***	*Apparent efficiency (%)*	*Observed counts*	*Apparent efficiency (%)*	*Observed counts*	*Apparent efficiency (%)*
[8-^{14}C]Hypoxanthine	258,761 ±1272	17,314	16,643 ±151	7.1	8099	6.9	3935	6.7
[8-^{14}C]Adenine	241,383 ±1632	16,149	15,882 ±213	7.2	7775	7.1	3799	6.9
[8-^{14}C]Adenosine	243,456 ±1964	16,287	15,797 ±169	7.1	7919	7.1	3897	7.0
[8-^{14}C]Deoxyadenosine	133,032 ±1135	8900	8043 ±127	6.7	4107	6.8	1961	6.5

* Sample dpm as determined by conventional liquid scintillation counting of collected peak (n = 5, ± S.E.M.).

** Observed counts as determined by radioactivity flow detector using peak integration program for ^{14}C.

TABLE III

APPARENT EFFICIENCIES FOR PURINE NUCLEOTIDES

Apparent efficiency calculated as described in text. Standard solution containing both radioactive and non-radioactive purines of known purity.

	Standard solution				*1:2 Dilution*		*1:4 Dilution*	
	*dpm_s**	*ECR*	*Observed counts***	*Apparent efficiency (%)*	*Observed counts*	*Apparent efficiency (%)*	*Observed counts*	*Apparent efficiency (%)*
[8-^{14}C]AMP	98,733 ±538	5615	5,503 ±58	6.1	3035	6.1	1528	6.0
[8-^{14}C]ADP	82,482 ±2405	3969	3,574 ±136	5.6	1775	5.6	862	5.2
[8-^{14}C]ATP	76,448 ±2635	3584	3,501 ±40	6.1	1788	6.0	783	6.1

* Sample dpm as determined by conventional liquid scintillation counting of collected peak (n = 5, ±S.E.M.).
** Observed counts as determined by radioactivity flow detector using peak integration program for ^{14}C.

with the calculated maximum apparent efficiency (6.25) for these operating conditions; although the values for the dinucleotide [8-^{14}C]ADP, were consistently lower than those observed for the mono- and trinucleotides.

Static counting efficiency was observed to change during the anion-exchange gradient. Determination of static efficiency was done in the same manner as for reversed-phase, except the gradient (0–80% B in A) was reconstructed in 10% increments. Fig. 2 shows that as ionic strength and pH increased (increasing % B in A eluent), the static counting efficiency decreased in a non-linear fashion. However, through use of this curve it was possible to identify the static efficiency for a specific point in the gradient. This value was then used in the calculation of the ECR for a compound eluted at that percentage of B eluent in the gradient.

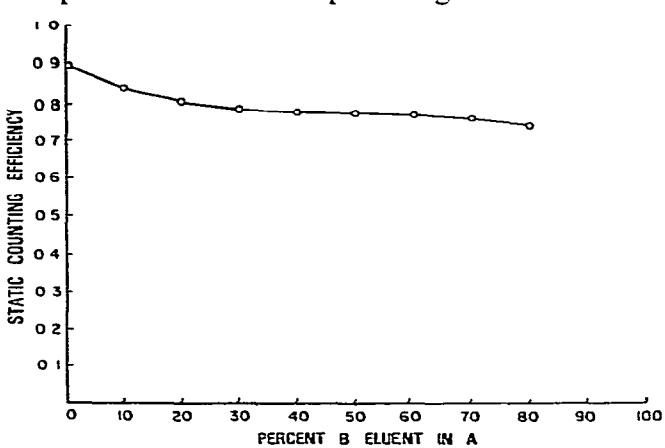

Fig. 2. Static counting efficiency for selected points in anion-exchange gradient. The gradient was reconstructed in 10% increments (0–80% B in A, over 30 min, linear) as described under Experimental. As ionic strength and pH increased (% B in A), the static counting efficiency decreased in a non-linear fashion.

Emphasis was placed on the measurement of ^{14}C-labelled compounds which possess high counting efficiency. The minimum detectable radioactive peak area (counts per peak) for quantitation with ^{14}C was approximately 100 cpm above background (^{14}C, 22 ± 8 cpm; ^{3}H, 17 ± 7 cpm; values represent mean ± S.E.M. for twenty determinations). This places the sensitivity at approximately $1 \cdot 10^{-3}$ μCi (≈2000 dpm) for a given peak under the described operating condition. However, for qualitative purposes, it was possible to consistently discern smaller radioactive peaks (≈ 20 cpm above background).

In addition, system performance for counting tritium (^{3}H) was examined for the reversed-phase separation of a purine base. Using the same procedures as described for ^{14}C, a static counting efficiency of 31 % was observed for [2,8-^{3}H]adenine ($1 \cdot 10^{-2}$ μCi/μl in 0.01 *M* KH_2PO_4, pH 5.6).

Application to the radioisotopic study of purine metabolism

To illustrate the application of this method to the study of purine metabolism, ^{14}C-labelled hypoxanthine was added to a flask of *P. falciparum* (malignant tertian malaria) grown in continuous erythrocyte culture. The purines were extracted in perchloric acid (PCA) and the neutralized PCA extract injected for HPLC analysis. Fig. 3 shows that the human malaria parasite was capable of using hypoxanthine for synthesis of both adenosine and guanosine nucleotides[14]. Excellent resolution of radioactive peaks was observed. The resolution for peaks 5–7 was particularly no-

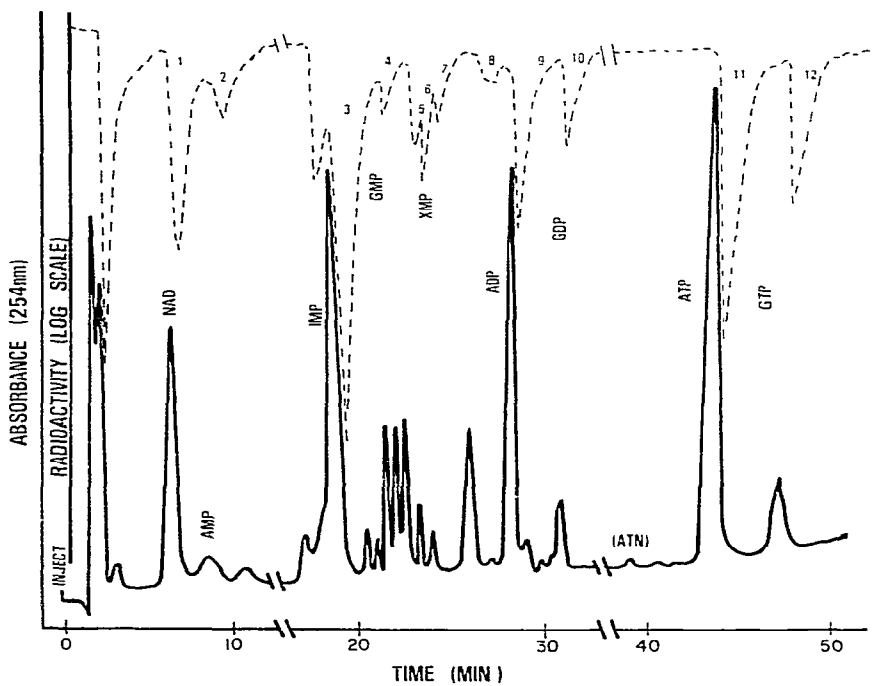

Fig. 3. Simultaneous UV–radioactivity HPLC anion-exchange separation of purine nucleotides in PCA extract of malaria infected human erythrocyte culture (48 h incubation, hematocrit = 36); [8-^{14}C]hypoxanthine (52.8 mCi/mmol) was added at the 24-h media change. Chromatographic conditions were as described in text.

table in that the presence of three peaks in this region of the chromatogram would probably have been missed by conventional fraction collecting and liquid scintillation counting techniques.

DISCUSSION

These procedures were developed specifically as a chromatography tool for the systematic study of purine metabolism in parasitic diseases leading to the development of new anti-parasitic chemotherapy. The rationale was based on the concept that the identification of critical or unique parasite metabolic pathways would allow selection of potential chemotherapeutic targets. The objective was to produce a dependable, sensitive and convenient method by which to follow the metabolism of added isotopically labelled purine precursors in cell culture. The procedures described in this study achieved the above criteria. Using the two HPLC separations in combination with the simultaneous UV–radioactivity detectors, it is possible to quantitatively define the major pathways of purine metabolism utilizing labelled purine precursors. The concise visual and quantitative features of the described chromatographic procedures give them a unique comprehensive value for the study of intermediary metabolic pathways.

Since this method measures both concentration and radioactivity of all major purine metabolites following incubation with a labelled precursor, the apparent rates of pathway enzymes can be calculated. This approach which involves summation of radioactivity in all purine metabolites along a defined branch of the purine metabolic pathway has been elegantly developed for application by thin-layer chromatography[15], and readily applies to the described HPLC procedures.

Overall, the dual detector system in combination with reversed-phase and anion-exchange HPLC provides a powerful tool for the systematic study of purine metabolism. Through the use of isotopically labelled purine precursors, metabolites or antimetabolites, it should be possible to obtain comprehensive information on the distribution of the radiolabel as well as the rate of metabolism for a given isotopic compound under various experimental conditions.

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