Journal of Chromatography, 343 (1985) 160—167
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2674

Note

High-performance liquid chromatographic detection of XMP as a basis for an improved IMP dehydrogenase assay

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(First received January 25th, 1985; revised manuscript received April 15th, 1985)

IMP, the first complete nucleotide formed during de novo purine nucleotide biosynthesis, is converted to GMP via the formation of XMP. The enzyme IMP dehydrogenase (IMP-NAD^+ oxidoreductase, EC 1.2.1.14) that catalyzes the conversion of IMP to XMP [1] has been well studied in several systems, since it catalyzes the first reaction unique to GMP biosynthesis. IMP dehydrogenase activity is most commonly determined by either monitoring the appearance of XMP by its differential absorbance at 290 nm compared to IMP, or by monitoring the reduction of NAD^+ at 340 nm [1]. Several radiometric assays have also been developed [2—4].

During our studies of the regulation of GMP biosynthesis in *Escherichia coli* K12, it is often necessary to determine low levels of IMP dehydrogenase activity in crude extracts. Under these conditions, the spectrophotometric assays are subject to interference from components of the crude extracts and they lack sensitivity. The radiometric assays, while more accurate, require that XMP be separated from IMP by high voltage electrophoresis [2] or thin-layer chromatography [3] before quantification. A more recently developed radiometric assay measures the release of ^3H_2O from IMP after oxidation by NAD^+ [4].

In order to improve the sensitivity as well as the specificity of the current assays, we have developed a high-performance liquid chromatography (HPLC) based method for the separation, identification and quantification of the IMP dehydrogenase assay components. To resolve XMP and IMP from each other and interfering compounds, we have employed a strong anion-exchange (SAX) column and a gradient elution procedure modified from the protocol of

0378-4347/85/$03.30

McKeag and Brown [5], a method which is a modification of the original protocol of Hartwick and Brown [6]. This method is particularly well suited to the determination of low levels of IMP dehydrogenase activity in crude extracts by monitoring the appearance of the product, XMP.

EXPERIMENTAL

Instrumentation

The HPLC apparatus used in these investigations consisted of the Gilson System 42 Gradient high-performance liquid chromatograph (Gilson Medical Electronics, Middleton, WI, U.S.A.) and included the following: 5-ml pump heads; a Rheodyne injection valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.) equipped with a 20-μl sample loop; two Spectroflow 773 absorbance detectors (Kratos, Ramsey, NJ, U.S.A.) (12 μl volume, 8 mm pathlength cuvette) one set at 260 nm, 0.030 absorbance units full scale (a.u.f.s.) and the other at 280 nm, 0.030 a.u.f.s.

All separations were performed at ambient temperatures (22–24°C) on a strong anion-exchange column (Partisil 10 SAX, 10 μm particle size, 25 cm × 4.6 mm I.D.) preceeded by a guard column (4 cm × 3.0 mm I.D.) packed with pellicular anion exchanger (37–53 μm particle size), and a precolumn (25 cm × 4.6 mm I.D.) packed with precolumn silica gel (Whatman, Clifton, NJ, U.S.A.).

Chemicals and reagents

IMP, GMP, XMP, NAD^+, glutathione (reduced), alkaline phosphatase (Type VII, calf intestine), potassium chloride (grade III), Tris and Sephadex G-25 were obtained from Sigma (St. Louis, MO, U.S.A.). Different lot numbers of potassium chloride must be tested to obtain one with minimal baseline rise in the course of the gradient. HPLC grade potassium dihydrogen phosphate was obtained from Fisher (Pittsburg, PA, U.S.A.). Distilled, deionized water used for the preparation of HPLC eluents and standard solutions was further purified by passage through a Norganic Cartridge (Waters Assoc., Milford, MA, U.S.A.). All HPLC solvents were filtered through 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A.) prior to use.

Preparation of crude extracts and IMP dehydrogenase assay

Crude extracts of various strains of *E. coli* K12 were prepared by sonication essentially as previously described [7], except that the crude extracts were desalted by passage through a Sephadex G-25 column [8]. IMP dehydrogenase was assayed by the method of Magasanik et al. [1] as described by Lambden and Drabble [9] at protein concentrations previously determined to be within the linear range of the assay. Samples were withdrawn from a central reaction mix at fixed time intervals, usually 10, 15 and 20 min, for the quantification of the amount of XMP formed.

After the IMP dehydrogenase reaction was terminated by boiling for 3 min, denatured proteins were removed by a 2-min centrifugation in a micro-centrifuge (Model 235A, Fisher). The supernatant was filtered through a 0.2-μm nitrocellulose filter (Schleicher and Schuell, Keene, NH, U.S.A.) by

centrifugation at 1500 *g* for 15 min in a clinical centrifuge (Model CL, IEC, Needham Heights, MA, U.S.A.). Samples were either injected immediately for HPLC analysis or frozen at −70°C, usually for two to three days, until assayed. A 20-μl aliquot was always injected for HPLC analysis by overloading the injection loop with 100 μl.

Preparation of standard curve

Working standard stocks (0.05—25 m*M*) of IMP and XMP were prepared by serial dilution in water from a 25 m*M* master stock. The standard curve points (0.005–2.5 m*M*) were prepared by diluting the working standard stocks (10% of final volume) into a Tris—hydrochloric acid—potassium chloride buffer mix of the same composition as the IMP dehydrogenase reaction mix. The Data Master was calibrated each day using a mid-range standard curve point. The 20-μl injection volume constituted a detection range of 100 pmol to 50 nmol for both IMP and XMP.

Peak identification and quantification

All peaks were identified on the basis of their retention time, co-chromatography with standards and comparison of the peak's dual wavelength ratio peak area at 260 nm/peak area at 280 nm with that of the appropriate standard. The peaks were also identified as nucleotides by enzymatic peak shift experiments with alkaline phosphatase. When control (zero time) IMP dehydrogenase assay mixes containing crude extract were analyzed, no endogenous peak was detected that would co-elute with XMP. The concentration of IMP and XMP in each assay sample was calculated using the external standard option on the Data Master. Depending on the expected activity of IMP dehydrogenase, the XMP external calibration standards used were: 0.03 m*M* (low) or 0.15 m*M* (high), both containing 1.50 m*M* IMP.

RESULTS AND DISCUSSION

The original procedure for the separation of nucleotides on a SAX column [6] was modified by McKeag and Brown [5] by the inclusion of potassium chloride in the gradient buffer, an increased flow-rate, and a raised pH of the high concentration eluent. We have further modified the gradient conditions to shorten the analysis times since we are interested in only a few of the nucleotides resolved under the original conditions. Our modification utilizes only the first third of the McKeag and Brown gradient [5], which resolves the following nucleotides: CMP, UMP, AMP, IMP, GMP, and XMP. The gradient conditions employed in this study for the resolution of IMP, XMP and GMP are described in Table I. The total time required to assay each sample was 35 min (22 min analysis and 13 min re-equilibration time). A representative chromatogram showing the separation of IMP, GMP and XMP standards under these gradient conditions is shown in Fig. 1. A 2.1% precision was obtained for the IMP and XMP peak area quantification as determined over a three-day interval (Table II).

In order to determine the minimal concentrations of IMP and XMP that we could detect with accuracy and linearity of detector response we ran standard

TABLE I

MODIFIED CONDITIONS OF McKEAG AND BROWN [5] GRADIENT ELUTION

Parameter	Condition
Low concentration eluent	0.007 *M* in potassium dihydrogen phosphate 0.007 *M* in potassium chloride
pH	4.0
High concentration eluent	0.125 *M* in potassium dihydrogen phosphate 0.250 *M* potassium chloride
pH	5.0
Flow-rate	2.0 ml/min
Isocratic elution, low concentration eluent	5 min
Gradient	Linear 0—98% in 17 min
Analysis time	22 min
Re-equilibration time	13 min
Total time required (analysis and re-equilibration time)	35 min

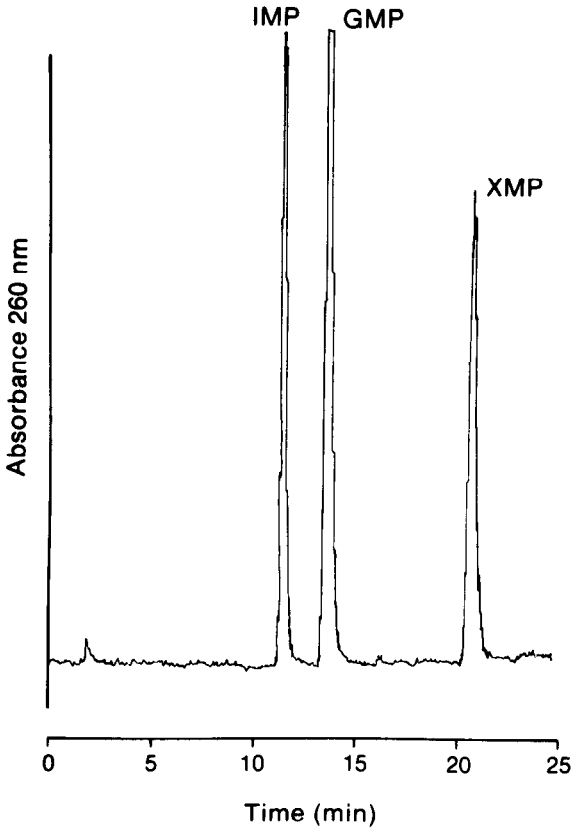

Fig. 1. Separation of IMP, XMP and GMP. A 20-μl aliquot of a 0.156 m*M* IMP, GMP and XMP solution (3.12 nmol of each compound) was injected. HPLC conditions are described in Table I. The detector was set at 260 nm and 0.030 a.u.f.s.

TABLE II

PRECISION OF IMP AND XMP QUANTIFICATION

	No. of samples	Relative standard deviation (%)
Peak area precision*		
IMP	6	2.1
XMP	6	2.1
Replicate assays**		
IMP	5	4.1
XMP	5	4.7

*Injections (20 μl) of a 1.5 m*M* IMP and 0.03 m*M* XMP calibration standard.
**Injections (20 μl) of a single assay point.

curves for both IMP and XMP. The detector response for IMP and XMP is shown in Fig. 2. We found that for both compounds the detector response was linear from 100 pmol to 50 nmol. The lower concentration ranges and detector response are shown in greater detail in the Fig. 2 inserts. We could easily detect 100 pmol of either IMP or XMP at 0.03 a.u.f.s. (the lowest detector setting is 0.001 a.u.f.s.). The accuracy could probably be improved by limiting the concentration range studied. This broad response range allows the development of additional enzyme assays, e.g., GMP synthetase and reductase, where all probable substrate and product concentrations could be quantified. We chose the external standard method of quantification by the Data Master, since we did not possess an appropriate internal standard which did not co-elute with one of the compounds of interest. The wavelength chosen for monitoring was 260 nm since XMP (the product of the reaction) has a 261-nm maximum in the low concentration buffer (Table I) while IMP exhibited a 248-nm maximum under the same conditions.

IMP dehydrogenase activity was assayed in crude extracts prepared from strains of *E. coli* K12 grown under various limiting or repressing guanine growth conditions. Fig. 3 shows the chromatograms of one such assay, which demonstrates the appearance and increase of XMP with time. As can be seen, XMP is well resolved from IMP, NAD^+, and various other compounds present from the crude extract. NAD^+ was not quantified due to its instability under our experimental conditions. The precision of replicate analyses from IMP and XMP quantification in crude extracts was 4.1% and 4.7%, respectively (Table II). This precision was calculated on an assay point run on two consecutive days. Using the HPLC method for the quantification of XMP, the crude extracts showed excellent linearity in the production of XMP over time. For six sample sets of four time points each, the correlation coefficient for the calculation of the specific activity of IMP dehydrogenase for each set was at least 0.996.

Confirmation of the identity of IMP (peak 2, Fig. 3) and XMP (peak 3, Fig. 3) peaks in the assay mix involved a two-fold approach. From the standard curve (n = 9), the mean dual-wavelength ratio (260/280) was 4.56 for IMP and 1.87 for XMP. The coefficient of variation was 6.3% and 9.9%, respective-

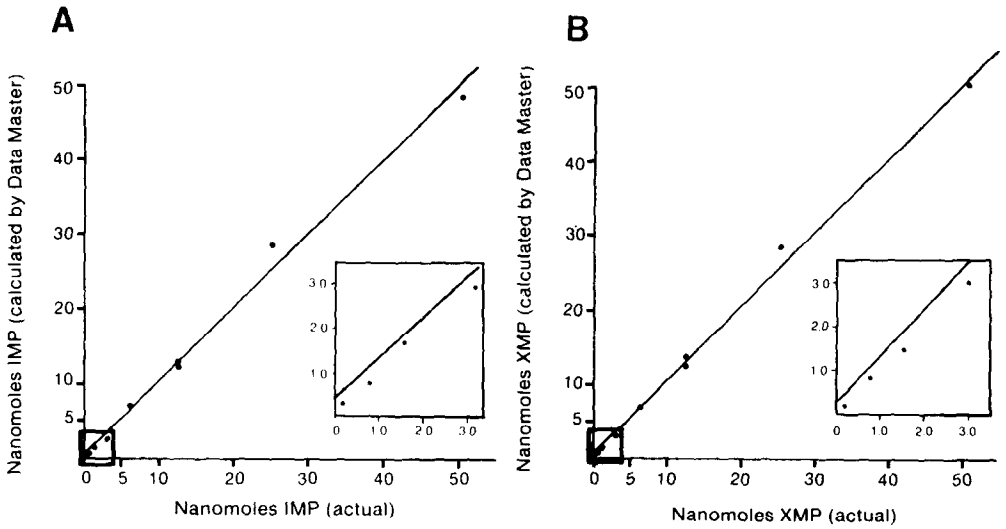

Fig. 2. Instrument response curves for (A) IMP and (B) XMP. The number of sample points n = 9 in both cases. The x axis represents the injected amount of IMP (A) or XMP (B), while the y axis represents the amount of IMP (A) or XMP (B) calculated by the Data Master. Chromatography conditions are described in Table I. The detector was set at 260 nm and 0.030 a.u.f.s. (A) Correlation coefficient = 0.9967; slope = 0.9934 and y-intercept = 0.463; (B) correlation coefficient = 0.9976; slope = 1.03 and y-intercept = 0.297.

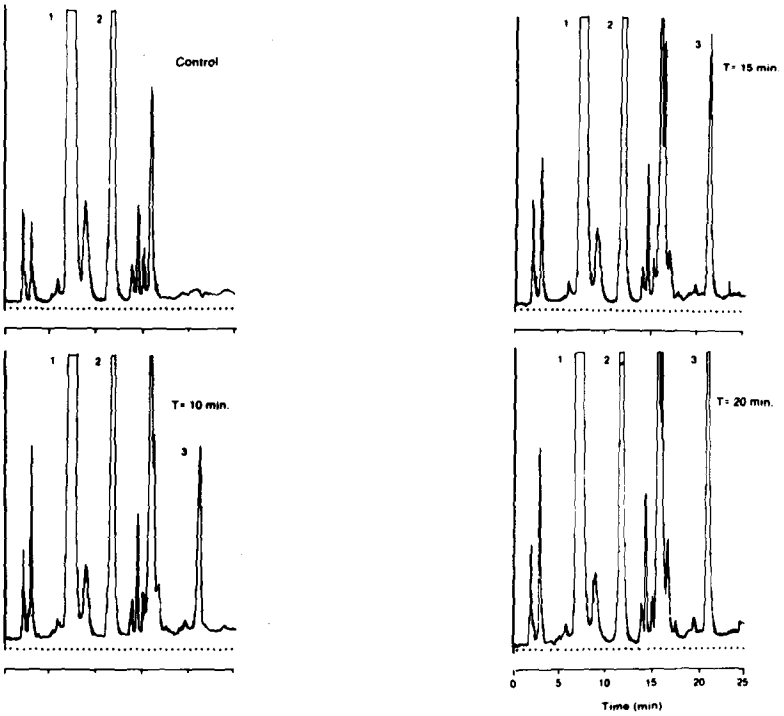

Fig. 3. IMP dehydrogenase activity as a function of time. NAD^+, IMP and the increase of the product XMP can be monitored. The control chromatogram represents the zero time point in the reaction. The detector was set at 260 nm and 0.030 a.u.f.s. Peaks: 1 = NAD^+; 2 = IMP; and 3 = XMP.

ly. The dual-wavelength ratio for peaks 2 and 3 of all assay points fell within the acceptable range of the ratio. Assay time points as well as standard curve mixes were also subjected to alkaline phosphatase treatment. Twenty-one units of alkaline phosphatase were added to 200 μl of the assay or standard curve points and incubated for 20 min at room temperature. Following termination by boiling, the alkaline phosphatase treated samples were subject to HPLC analysis under our standard conditions. The IMP and XMP peaks were absent and a new, large peak was present in the void volume where nucleosides would elute.

To determine if any loss of IMP and XMP occurred during the filtration step of the sample clean up procedure, the standard curve points were used to spike complete assay mixes that lacked IMP and XMP. No loss of IMP or XMP could be detected at the range of concentrations used or expected in the assay mix. Any potential losses of IMP or XMP could be circumvented by analyzing the deproteinated assay points directly.

A control containing all possible components of the incubation mix, except for IMP, showed a small endogenous peak which coeluted with IMP. The area of this peak was less than 3% of the area of the IMP peak. When IMP was monitored, there was a correlation between the decrease in IMP and the increase of XMP if the amount of XMP produced was 15% or more of the initial IMP concentration. Thus, in crude extracts with high IMP dehydrogenase activity, both the disappearance of IMP and appearance in XMP could be monitored to determine the activity. At low levels of IMP dehydrogenase activity, the small decrease in a large IMP concentration could not be accurately measured because of the percentage error inherent in the precision of the method and the contribution of the small endogenous peak that coeluted with IMP. This does not affect the validity of the assay since the measurement of the product XMP is the most valid assessment of IMP dehydrogenase activity.

Under our assay conditions we did not detect the conversion of XMP into any other product. Conversion of XMP into GMP did not occur since the necessary cofactors were not present and the Sephadex G-25 chromatography [8] removed any endogenous cofactors present in the crude extract. This was also indicated by the correlation of the decrease of IMP with the increase of XMP.

The IMP dehydrogenase activity in each crude extract was also determined by the spectrophotometric method (absorbance at 290 nm). When the results were compared, the specific activity as calculated from the HPLC data was approximately half the value calculated from the spectrophotometric method. We are unable to assign a specific reason for this difference, other than to point out that the HPLC analysis specifically measures XMP formation while the spectrophotometric method is subject to interference by components of the crude extract.

The sensitivity of this HPLC method can be further increased either by injecting a larger sample volume, or by lowering the range setting of the detector up to ten-fold. Of course, this assay could be directly converted to a radiometric assay for the most advantageous combination of specificity and sensitivity. The advantage of this method is its applicability to the determina-

tion of low levels of IMP dehydrogenase activity in crude extracts, as well as its greater sensitivity and specificity compared to the spectrophotometric method.

ACKNOWLEDGEMENTS

This study was supported by NIH Grant AI 20068. We appreciate the assistance of Steve Wolfe in the preparation of the crude extracts and the production of the spectrophotometric data, and Carol Ann Ardoin's skillful typing of this manuscript.

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