

A MODIFIED APPROACH FOR RAPID DETERMINATION OF THE QUALITY OF INDUSTRIALLY PRODUCED P-33 ORTHOPHOSPHORIC ACID PREPARATIONS

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In this work we have developed an approach to rapidly estimate the quality of P-33 labeled Orthophosphoric Acid (carrier free) in industrial production. The method was created to determine the values of specific radioactivity for both Orthophosphoric acid and gamma-Adenosine 5'-Triphosphate (ATP) by means of enzymatic ATP synthesis in equal molar ratio conditions for two main substrates (radioactive Orthophosphoric acid and ADP) with following chromatography of the reaction mixture on the PEI-cellulose micro column.

INTRODUCTION

As it is known, the standard method for the quantitative determination of the specific radioactivity for P-32 or P-33 labeled Orthophosphoric Acids (carrier free) is based on isotope dilution and inorganic Vanadate-Molybdate complex formation. The specific radioactivity for gamma-ATP can be determined from the reverse phase chromatography of the labeled compound, followed by a calculation of the ratio of the radioactivity to the molar quantity of ATP in the peak. Usually a many-fold excess of ADP is added into the reaction mixture to prepare the gamma-ATP by means of enzymatic synthesis [1, 2, 3]. So, depending on the quality of the ADP, beta-NAD and other chemicals that are used in the synthesis, there can be considerable isotope dilution of the initial labeled Orthophosphoric Acid. Therefore the specific radioactivity of gamma-ATP that is produced from commercial labeled Orthophosphoric Acid (carrier free) probably does not correspond to the real value.

Recently we have proposed a method that at once permits determination of the specific radioactivity for both labeled Orthophosphoric Acid and gamma-ATP; one to measure the of biological activity for Orthophosphoric Acid (percentage of incorporation into gamma-ATP); and avoid the discrepancies mentioned with customers. This can be achieved by means of high yield enzymatic ATP synthesis under equal molar ratio conditions of the main participants (radioactive Orthophosphoric acid and ADP), followed by chromatography of the reaction mixture in a PEI-cellulose micro column.

MATERIAL AND METHODS

The following chemicals and reagents were purchased from Sigma (USA): Glycophosphate Dehydrogenase, Triosephosphate Isomerase, Glyceraldehyde-3-Phosphate Dehydrogenase, Phosphoglycerate Kinase, Lactate Dehydrogenase, ADP, beta-NAD, Trisma base, DTT, M-L- α -Glycophosphate, Di-monocyclohexylammonium Salt,

Sodium Pyruvate, Polyethyleneimine-Cellulose (fine mesh), PEI-Cellulose for TLC, Potassium Phosphate (KH_2PO_4), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and Lithium Chloride. P-33 Orthophosphoric Acid preparations were routinely produced. Radioactivity was measured by means of a Rack Beta (LKB, Sweden) scintillation counter.

Enzymatic synthesis of gamma-ATP

1. Prepare the enzymes mixture in the form of Ammonium Sulphate residues: 21.6 units of Glycerophosphate Dehydrogenase, 20 units of Triosephosphate Isomerase, 36 units of Glyceraldehyde-3-Phosphate Dehydrogenase, 36 units of Phosphoglycerate Kinase, 130 units of Lactate Dehydrogenase. Following centrifugation, dissolve the sediment in the buffer solution.
2. 1/7 part of the enzymes mixture should be centrifuged at 12000 RPM, for 2 minutes. Following centrifugation, dissolve the sediment in 300 mkl of a buffer consisting of 0.1 M Tris-HCl, at pH 9.0, and 0.01 M DTT.
3. One then prepare a solution of the following: 150 mkl of 0.5 M Tris-HCl, at pH 9.0; 30 mkl of 1 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 90 mkl of 0.1 M DTT; 90 mkl of 0.012 M L- α -Glycerophosphate Di-monocyclohexylammonium salt; 450 mkl of 0.01 M β -NAD, 450 mkl of Sodium Pyruvate; 300 mkl of enzymes solution prepared above (Item 2).
4. The following is mixed for P-33 gamma-ATP synthesis: 50 mCi of P-33 Orthophosphoric acid (neutralized by Trisma base or in water solution); 150 mkl of reaction mixture (from item 3); and 2, 5, 10, 20, 50 or 100 nmol of ADP in water solution. The mixture is incubated at room temperature for 30 minutes.

Detection of P-33 Orthophosphoric acid incorporation

Distribution of radioactivity was detected by means of a TLC on PEI-cellulose covered plate in a 0.5 M KH_2PO_4 system for each reaction mixture containing different quantities of ADP.

Micro column chromatography of reaction mixtures

Chromatography was performed using LKB equipment on the PEI-cellulose packed micro column (2.0 x 50 mm) with a stepped gradient of LiCl in 20 mM Tris-HCl, pH 7.5. Optical density was detected by absorbance at 254 nm.

RESULTS

Gamma-P-33 ATP synthesis

It should be specially emphasized that mass produced P-33 Orthophosphoric acid preparations are carrier free. That means 1 mCi of P-33 Orthophosphoric acid must contain 0.2 nmol of the compound. For the convenience of the following discussion, see Table 1, which shows the principle data for the experiment and the incorporation of radioactivity into gamma-P-33 ATP for different quantities of ADP. The data confirms the trend where increasing the amount of P-33 OPA proportionally increases incorporation until it is the same as the amount of ADP present and after which the incorporation saturates. A more visual illustration is shown in Figure 1. Note the

strong bend point in the figure, at the end of the linear portion of the curve, corresponding to point where the fraction of incorporation begins to saturate.

Table 1. *Incorporation of P-33 Orthophosphoric acid radioactivity into gamma-P-33 ATP for different quantities of ADP*

P-33 OPA quantity, mCi nmol	50 10	50 10	50 10	50 10	50 10	50 10
ADP quantity, nmol	2	5	10	20	50	100
Average incorporation, %	12.1	48.4	96.5	97.8	98.4	98.7

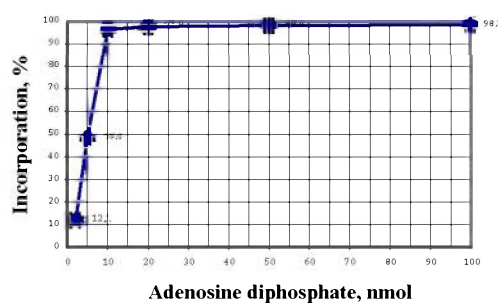


Figure 1. Incorporation of P-33 Orthophosphoric Acid (50 mCi for each point) radioactivity gamma P-33 ATP depending on ADP quantities

Micro column chromatography

Figure 2 shows the elution of reaction mixture pattern for equal molar ratio conditions of radioactive Orthophosphoric Acid and ADP. Position of the ATP peak corresponds to most of the radioactivity: it contained 96.5% of the radioactivity applied to the column. At the same time, the chromatography of the pattern with two-fold excess of ADP demonstrates the appearance of an ADP peak at the above-mentioned value of P-33 Orthophosphoric Acid incorporation into ATP (see Figure 3).

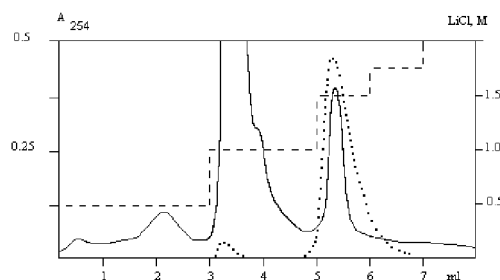


Figure 2. Chromatography of the reaction mixture containing equal molar ratio of γ -P-33/ Orthophosphoric Acid (25 mCi, 5 nmol) and ADP (5 nmol). 2.0 x 50 mm PEI-cellulose micro column, the elution with 7 ml stepped gradient of LiCl in 20 mM Tris-HCl, pH 7.5, 30 ml/h, 0.5 ml fractions.

_____ A_{254} , ----- stepped gradient LiCl, radioactivity.

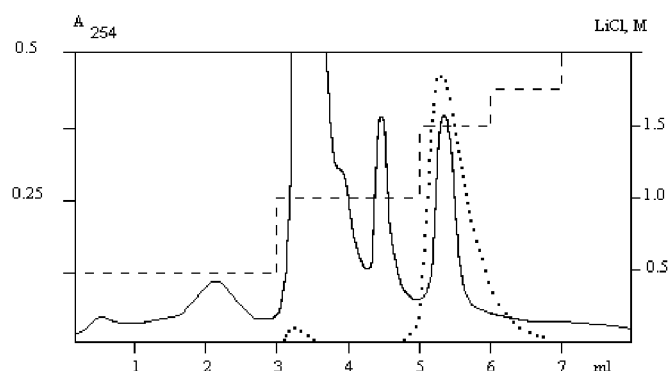


Figure 3. Chromatography of the reaction mixture containing 2-fold molar deficiency Acid (25 mCi, 5 nmol) and ADP (10 nmol). 2.0 x 50 mm PEI-cellulose micro column, the elution with 7 ml stepped gradient of LiCl in 20 mM Tris-HCl, pH 7.5, 30 ml/h, 0.5 ml fractions. The solid line (—) denotes A_{254} , the dashed line (---) denotes the stepped gradient LiCl, and the dotted line (·····) denotes the radioactivity.

DISCUSSION

The method described in this paper has made it possible to estimate most of the necessary characteristics of the specifications for manufactured batches of P-33 Orthophosphoric Acid preparation by means of a one step analysis.

First, this approach allows one to determine the values of specific radioactivity for both labeled Orthophosphoric Acid and for P-33-gamma-ATP and to effect measuring of biological activity for Orthophosphoric acid (percentage of incorporation into gamma-ATP)—simultaneously. The above evidence demonstrates very high efficiency of gamma-P-33 ATP synthesis under equal molar ratio conditions for Orthophosphoric Acid and ADP. Under these conditions, the labeled gamma-ATP has the same specific radioactivity as P-33 Orthophosphoric Acid.

Secondly, the accuracy of the approach is far higher than in the method based on isotope dilution combined with inorganic Vanadate-Molybdate complex formation.

The advantages of the method described here are the following: (a) it is very rapid, where the final result can be received during one hour; (b) it is simple and does not depend on any expensive equipment; (c) it gives the complete confirmation of the best quality for industrial produced preparations of P-33 Orthophosphoric Acid.

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