DETERMINATION OF MONOPHOSPHATE-TYPE NUCLEOTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOSPHORUS-SELECTIVE DETECTION

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Abstract - A selective, method for the determination of monophosphate-type nucleotides based on the molybdenum blue method has been developed. The monophosphates were decomposed to produce the orthophosphate-molybdenum blue complex by heating at 140 °C without any oxidizing agent, while di- or triphosphates hardly produced the complex under the same condition. The separation of monophosphates was carried out in the ion-exchange mode, and the detection limits were 25 ppb as P for the monophosphates examined.

Key words monophosphate-type nucleotides, molybdenum blue method, selective detection, ion-exchange mode-HPLC

A phosphorus-selective detection method was developed for the determination of nucleotides in our previous work [1]. The method was based on reversed-phase ion-pair high-performance liquid chromatography (HPLC) in combination with the post column molybdenum blue reaction. In the previous work [1] the effluent from the separation column was mixed with potassium peroxodisulfate oxidizing agent to produce orthophosphate, and then the orthophosphate was converted to the phosphorus-molybdenum blue complex, followed by visible absorption detection at 880 nm. Any type of nucleotides could be detected by the previous method. The present paper will propose a selective detection method for only monophosphate-type nucleotides.

EXPERIMENTAL

Apparatus The chromatographic system employed in this work was the same as in the previous work [1] except that the volume of the loop injector was changed to 5 μ l in this work. Develosil NH₂-5 (250 x 4.6 mm ID, 5 μ m; Nomura Chemical, Seto, Japan) was employed as the separation column. The eluent from the separation column was mixed with a complexation reagent containing 2.5% (W/V) ammonium molybdate, 0.36% (W/V) L-ascorbic acid and 1.5 M sulfuric acid, and passed through a 8 m x 0.5 mm ID PTFE reaction coil heated in an aluminum block bath at 140 °C where the hydrolysis and complexation reaction simultaneously took place. The molybdenum blue formed was then monitored by a UV detector at 880 nm.

Reagents All the reagents were of reagent-grade and obtained from Wako (Osaka, Japan) and Sigma (St. Louis, Mo, USA). Adenosine-5'-monophosphate (AMP), adenosine-5'-diphosphate (ADP) and adenosine-5'-triphosphate (ATP) were obtained from Wako, and cytidine-5'-monophosphate (CMP), inosine-5'-monophosphate (IMP), guanosine-5'-monophosphate (GMP), guanosine-5'-diphosphate (GDP), guanosine-5'-triphosphate (GTP) and uridine-5'-monophosphate (UMP) were obtained from Sigma. The mobile phase was citric buffer (4 mM, pH 5.3), and the flow rate was 0.5 ml/min. The flow rate of the complexation reagent was kept at 0.05 ml/min. The concentration of the nucleotides are given in ppm as the phosphorus atom throughout the text, unless otherwise noted.

RESULTS AND DISCUSSION

Because it is orthophosphate that forms the complex with molybdate in the molybdenum blue method, condensed phosphoric acids and organophosphorus compounds must be decomposed to orthophosphate before the complexation. For this purpose, potassium peroxodisulphate was employed as the oxidizing agent [2]

The effect of the oxidizing agent on the signal intensity was examined. We have found that AMP can be decomposed to orthophosphate by the hydrolysis reaction at temperature higher than 110 °C without any oxidizing agent and that no response is appeared when the reaction temperature is kept at lower than 90 °C. The result is shown in Fig.1. The signal intensity was increased with increasing reaction temperature, and at about 140 °C the signal intensity was the same as that of orthophosphate with the same concentration. This means that AMP can be completely decomposed to produce orthophosphate by the hydrolysis reaction at 140 °C without any oxidizing agents. For another monophosphates the same results were obtained at the reaction temperature of 140 °C. Therefore, the reaction temperature was kept at 140 °C.

Fig.1. Dependence of the signal intensity on the reaction temperature.
Column: Develosil NH₂-5, 250 x 4.6 mm ID, 5 μ m. Mobile phase: citric buffer, pH 5.3. Flow-rate of mobile phase: 0.5 ml/min. Flow-rate of complexation reagent: 0.05 ml/min. Visible wavelength: 880 nm. Analytes: 50 ppm AMP.

TABLE 1. Recoveries of nucleotides.
Reaction temperature: 140 °C

Compounds	Monophosphates					Diphosphates		Triphosphates	
	AMP	CMP	GMP	IMP	UMP	ADP	GDP	ATP	GTP
Recovery(%)	100	100	101	101	100	3.3	2.1	0	0

Table I shows the recoveries of nucleotides when the reaction temperature is kept at 140 °C. The results in Table I indicate that the monophosphate-type nucleotides are almost completely decomposed to produce orthophosphate by

the reaction at 140 °C, while di- or triphosphates were hardly decomposed, e.g., the recoveries of diphosphates are less than 3.3%, and no signals were observed for the triphosphates.

Response signals for the monophosphate-type nucleotides were examined. The single calibration graph was required for all the monophosphates examined, viz., AMP, CMP, GMP, IMP, and UMP, if the peak area was plotted versus the concentration of phosphorus. This suggests that the conversion of the monophosphates examined to orthophosphate is complete. The calibration graph was linear up to 150 ppm.

Fig.2 shows the selective detection of AMP. in which an artificial mixture of analytes such as 50 ppm AMP, 100 ppm ADP. and 100 ppm ATP was injected. Under this condition, ADP was still detected as predicted from the results shown in Table 1.

Fig.2 Selective detection of AMP
Operating conditions as in Fig.1 except the analyte: 50 ppm AMP. 100 ppm ADP. and 100 ppm ATP. Reaction temperature:140 °C.

Another example of selective detection of monophosphates is shown in Fig.3, in which five kinds of monophosphates are injected. The detection limits for all the monophosphate-type nucleotides were 25 ppb as P under the operating conditions in Fig.3.

Fig.3 Selective detection of monophosphates.
Operating condition as in Fig.2 except the analytes. Analytes: 30 ppm CMP. 70ppm UMP. 25 ppm AMP. 65 ppm IMP and 20 ppm GMP.

CONCLUSION

Monophosphate-type nucleotides could be selectively detected based on the molybdenum blue method without any oxidizing agent. Selectivity will be further improved by the optimization of the reaction conditions.

REFERENCES

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