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Separation of Cytidine 5′-triphosphate Biosynthesized from Cytidine 5′-monophosphate on Ion-exchange Resin and HPLC Analysis of Cytidine Compounds

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Abstract Conditions were studied in the biosynthesis of cytidine 5′-triphosphate (CTP) from cytidine 5′-monophosphate (CMP). A 201×7 anion ion-exchange resin was applied for the separation of CTP from CMP. Adsorption isotherm and elution conditions (eluant, eluant concentration, flow rate, sample volume loaded) were investigated. At the same time, a new high-performance liquid chromatography on an anion ion-exchange column WAX-1 with UV detector at 260 nm was developed to measure CMP, cytidine 5′-diphosphate (CDP), and CTP. The retention time for CMP, CDP, and CTP are 0.723, 1.448, and 4.432 min, respectively. This new rapid high-performance liquid chromatography (HPLC) method for the analysis of cytidine compounds in biological sample has a wide linear range with high precision and repeatability.

Keywords Biosynthesis · Cytidine 5′-triphosphate · Anion ion-exchange resin · HPLC · Cytidine 5′-monophosphate

Introduction

Cytidine 5′-triphosphate (CTP) plays an essential role in the growth and metabolism of all organisms. It promotes intracellular synthesis of nucleic acids [1], protein, and membrane phospholipids [2], and regulates synthesis and reconstruction of neurolemma, especially the metabolism of components in synapse. Therefore, CTP is nutritious to neurons and delays

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their death. It also increases neuron activity and the resistance against injury as well as facilitates growth of neuron process. CTP is predominantly used in curing cerebrovascular accident and its sequelae, brain concussion, cerebral arteriosclerosis, senile dementia, and diabetic peripheral neuropathies [3]. CTP is also the direct precursor of cytidine 5′-monophosphate (CMP)-NeuAc [4], cytidine 5′-diphosphate (CDP)-choline, and CDP-ethanolamine.

Qiu reported the manufacture of CTP by immobilized yeast [5], but he did not mention the conversion ratio. Zhan et al. [6] reported the synthesis of CTP by photophosphorylation and the conversion ratio of which was 85%. Simon et al. investigated the generation of CTP from CMP using adenylate kinase with 90% conversion in 9 days [7]. This method cost too much time and the cost of enzyme was also high. Hong et al. [8] reported biosynthesis of CDP-choline by immobilized beer yeast cells with a yield of 40%. In this paper, CTP was biosynthesized from CMP using enzyme extracted from discarded beer yeast cell. The conversion ratio was nearly 90% in 2 h. This method provides the most convenient route to produce CTP with less cost. If CMP is available, synthesis of CTP can be accomplished rather easily.

Purification of CTP from the reactants is a more difficult task. Jiang et al. [9] reported the separation of CDP from CMP on anion ion-exchange resin, and the conditions were not mentioned. Zhu et al. [10] studied the adsorption kinetic and the thermodynamic study of CTP gelation duolite. No literature has reported the purification of CTP from CMP by an anion ion-exchange resin. In this paper, the most suitable resin was selected and the optimal purification conditions of CTP from CMP on resin 201×7 were established.

A firefly luciferase assay for determination of CTP in biological sample was studied [11]. Ozier-kalogeropoulos [12] reported the analysis of CTP by thin-layer chromatography on poly(ethylenimine)-cellulose, which was commonly used to determine nucleotides. However, the former two methods cost too much time, and the precision was not so good. The operation was also complicated. Capillary electrophoresis was also applied to separate common nucleotides, mono-, di-, and triphosphates [13]. Although literature has described the detection of nucleotides by high-performance liquid chromatography (HPLC) [14–17], to the best of our knowledge no paper aimed to separate the cytidine compounds. In this paper, a simple and fast HPLC method was developed. The whole detection course can be accomplished within 7 min.

Experimental

Reagents

CMP, CDP, CTP were purchased from Sigma (the purity was more than 90%). Beer yeast cell was a gift from XiLing Beer (HangZhou, China). The resins used in this study were purchased from ShuangLin Chemical reagent factory (HangZhou, China). Other chemicals used in this study were of analytical grade.

Enzyme Extract from Beer Yeast Cell

The enzyme needed in the biosynthesis from CMP to CTP was extracted from inutility beer yeast. A 20-g beer yeast, after being frozen–melted three times, was put into a 250-ml Erlenmeyer flask with 20-ml culture (glucose 8.0 %, ammonium sulfate 0.4 %, 0.3 % potassium dihydrogen phosphate, 0.1 % magnesium chloride, *w*/*v*). The culture was

incubated at water bath at 37°C for 2 h. Then it was centrifuged at 3,000 rpm for 10 min. The supernatant thus obtained was used as the crude enzyme preparation.

Biosynthesis of CTP from CMP

A 250-ml Erlenmeyer containing 20-ml enzyme preparation, 60-mM CMP, 250 mM sodium phosphate buffer (pH 7.0), 8 mM magnesium chloride, 150 mM glucose, and 3 mM adenosine triphosphate were incubated at 35°C for 2 h. The reaction was terminated by the addition of 2 ml trichloracetic acid (40%, *v*/*v*). Thermal deactivation was given to the mixture at 90°C in water bath for 10 min to eliminate unwanted proteins. Then it was centrifuged at 6,000 rpm for 15 min. The precipitation was discarded and the supernatant thus obtained was used for further purification.

Isolation of CTP by Ion-Exchange Resins

The resin used in this study, 201×7, was a strong anionic exchange resin, based on cross-linked polystyrene matrix. The physical property of it was summarized in Table 1.

Before any experiments, all the resins were washed with ethanol to remove any impurities. And then the resins were pretreated by repeated washes with 2 M HCl and 2 M NaOH solutions, and then converted to the hydroxide form by elution with 2 M NaOH and rinsed at neutral pH with deionized water. Saturation experiments were carried out using an Omnifit glass column (10×30 mm) filled with resin particles. A peristaltic pump (Ismatec, model ISM 832) was used to percolate the solution through the column. Before starting each experiment, the resin in OH form was equilibrated with deionized water. A solution containing a given initial concentration of CTP was introduced at the entrance of the column as a step input. The concentration of CTP at the outlet column along the time was measured by HPLC as described below.

The concentration of sodium chloride was determined by specific conductance by a conductance apparatus. The solution pH was determined by a pH meter (WTW 540 GLP) using a glass combined electrode (Mettler, Toledo).

Adsorption Isotherm

Ion exchange isotherms were obtained at pH 6.0 and 3.0 from batch experiments, respectively. Varying amounts of dry resin, in hydroxide form (anion exchanger resins), were placed in shaking Erlenmeyer flasks in contact with solutions (25 ml) containing known initial concentration of standard CTP. The flasks were sealed and shaken for 12 h (time required to reach equilibrium) in a constant-temperature bath (Julabo SW-21C) at 25°C. After

Table 1 Physical properties of resin 201×7.

Ionogenic group	$-N^{+}(CH_3)_3$
Degree of cross-linking (%)	7
Ion exchange capacity (meq/g)	3.5
Intraparticle porosity	0.64
Water content (%)	45
Density ($g_{wr}/cm^3_{\ wr}$)	1.01
Name of basic functional group	Strong

equilibrium was reached, solution in the flask was sampled to determine the residual CTP concentration. The equilibrium concentration of CTP in the resin phase was determined from material balances.

Desalting of CTP

The CTP fractions collected from the ion-exchange column were pooled and concentrated. Then they were dialyzed against 20 mM sodium phosphate buffer (pH 7.0) at 4°C for 24 h.

Chromatographic Conditions

The concentration of CMP, CDP, and CTP were determined by HPLC with N_{2000} system (Thejiang University, China). High-performance liquid chromatography was carried out on a Shim-pack WAX-1 column (50×4 mm, 5-μm particle size, Shimadzu, Japan) using UV detector (SPD-10A, Shimadzu, Japan) at 260 nm at a flow rate of 1 ml/min. The mobile phase was a 480 mM potassium dihydrogen phosphate solution (pH 5, adjusted with 100 mM NaOH). The ambient temperature was around 25°C and not specifically controlled. Injection volumes possible in the system were 5–50 μl; typically, however, a sample volume of 10 μl was used. A sensitivity setting of 0.01 and a time constant of 7 min were settled.

Results and Discussion

Biosynthesis Conditions of CTP from CMP

CTP was synthesized enzymatically in the following two steps, as depicted in Fig. 1. In this work, enzyme was not purified, just the crude one extracted from beer yeast cell. During the biosynthesis process, several factors affected the conversion ratio of CTP from CMP. Concentrations of CMP, sodium phosphate buffer, magnesium chloride, ATP, and glucose were studied in this work. At the same time, enzyme preparation volume, reaction time, and solution pH were also investigated. As a result, the concentration of ATP and magnesium chloride had no effect on the conversion (data not shown). The literature [1] reported Mg^{2+} and ATP were absolutely necessary in the reaction. It was said that Mg^{2+} was the activator of the reaction from CMP to CTP. Maybe the concentration of Mg^{2+} existing in the enzyme preparation was enough to activate the reaction. ATP provided energy to any biosynthetic reaction. Maybe ATP could be produced through glycolysis pathway in the existence of glucose, so the reaction does not need the extra addition of ATP. Reaction time, solution pH, concentrations of CMP, sodium phosphate, and glucose had a marked effect on the conversion as shown in Fig. 2a–e.

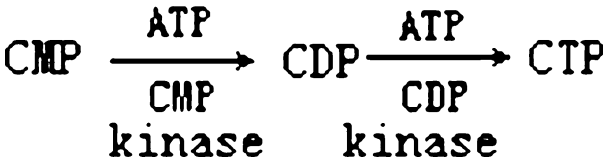

Fig. 1 The biosynthesis step of CTP

As seen in Fig. 2a, it had a same optimal reaction time of 2 h with different concentrations of substrates. The optimal concentration of substrate was 40 mM. When CMP concentration was 40 mM, the conversion was higher than that of other concentrations all the while. Nearly 90% CMP could be converted into CTP when its concentration was 40 mM. From 0 to 40 min, 20 mM substrate had the same conversion ratio with that of 40 mM. However, it had a lower conversion ratio than that of 40 mM with

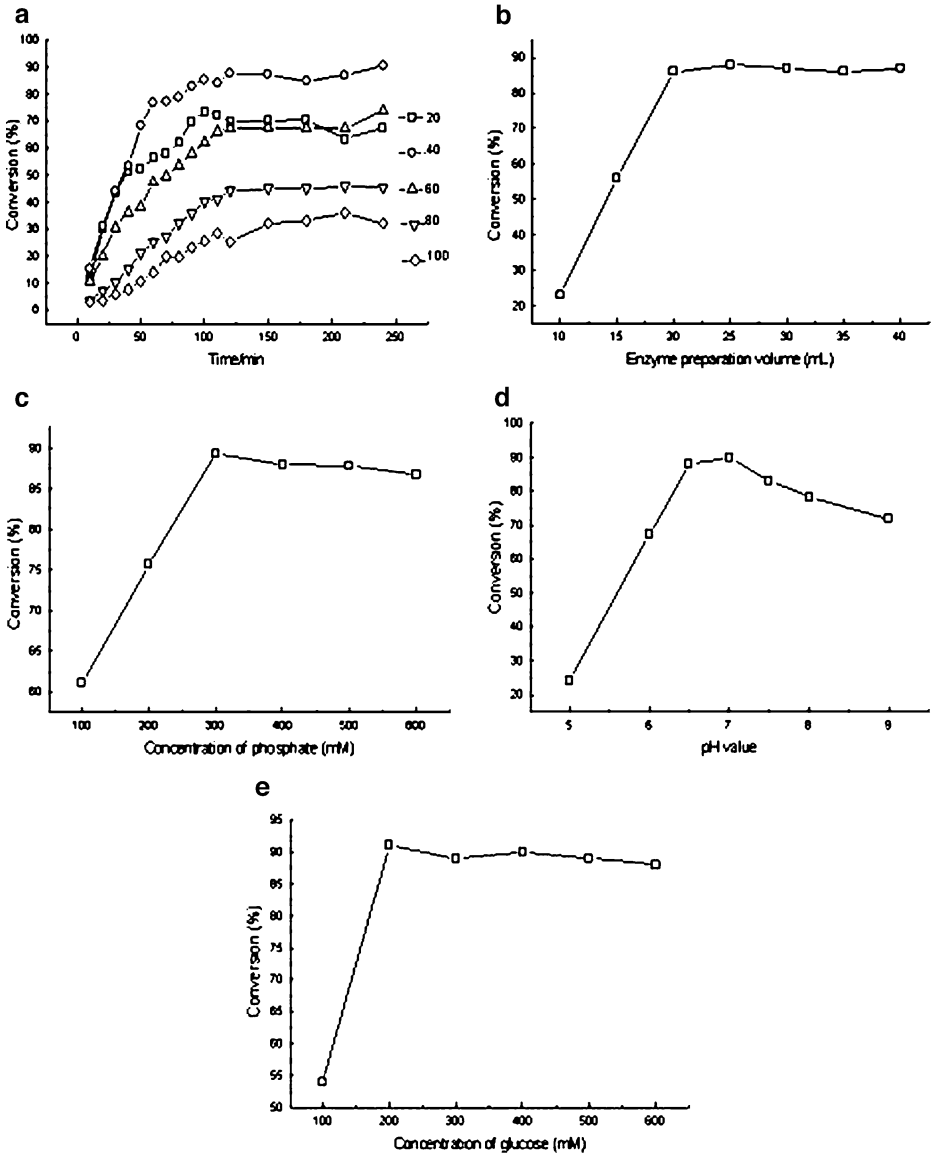

Fig. 2 (**a**) The effect of reaction time and substrate concentration on the biosynthesis CTP. The unit of the substrate (CMP) was mM. (**b**) The effect of enzyme yield on the biosynthesis of CTP. (**c**) The effect of glucose concentration on the biosynthesis of CTP. (**d**) The effect at pH value on the biosynthesis of CTP. (**e**) The effect of glucose concentration on the biosynthesis of CTP

the increasing reaction time all along. Further, 20-mM substrate concentration reduced the yield of CTP. Although a high-substrate concentration could lead to more CTP, the conversion ratio was reduced with the increase of substrate concentration. Taking into account the economical cost, 40-mM CMP as the initial substrate concentration was appropriate.

Monomolecular CMP converted into monomolecular CTP needed dimolecular phosphate. Phosphate was obligatory in CTP synthesis process. Furthermore, phosphate could be catalyzed to form glucose phosphate compound during the glycolysis pathway, at the same time to motivate the existence of ATP and release phosphate anion for the phosphorylation reaction. Phosphate was important to the biosynthesis of CTP. It was obvious that the conversion ratio was enhanced with the increase of sodium phosphate concentration (Fig. 2c). On the other hand, the conversion ratios were much the same when phosphate concentration was above 300 mM. Perhaps 300 mM phosphate was enough to the phosphorylation reaction. Exorbitant phosphate would not repress the reaction, so it was not necessary to control the concentration of phosphate so far as it reached 300 mM.

As seen in Fig. 2b and Fig. 2e, it was also unnecessary to control the concentration of glucose and enzyme so far as they reached a certain amount. It was said that excess glucose would go against the reaction. The conversion ratio was not affected by the concentration of glucose when it reached 200 mM. Normally, more enzyme will lead to more production. As seen in Fig. 2b, the conversion ratio was increased with the increasing amount of enzyme until the volume was 20 ml. From then on the conversion ratio was much the same in spite of the increasing enzyme amount. The reaction was critical to pH value. The effect of pH was shown in Fig. 2d. The conversion ratio was increased when pH value was from 5.0 to 7.0. The maximum conversion ratio could be obtained under pH 7.0. When pH was above 7.0, the conversion ratio was reduced all the time, so it was necessary to control the initial pH value in the reaction.

Adsorption on Different Resins

It is known that CTP solution has a negative charge when it is in the neutral pH. This is because it has three phosphate anions within itself. Therefore, it is better to choose strongly alkalic anion ion exchange resins to separate CTP from CMP. 201×7, 201×8, 201×4, D201, and D202 were applied to select the optimum to separate CTP, which were all based on cross-linked polystyrene matrix. Results are shown in Table 2. It was obvious that 201 series had greater adsorption capacity than D201 and D202 had, which were in the macroreticular form. 201×7 and 201×8 had the same function groups even with different cross-linking. Therefore, they almost had the same adsorption capacity, whereas the

Table 2 The effect of different resins.

Resin	The maximum capacity of resin (mg/g)	Recovery (%)
201×4	139	80
201×7	153	80
201×8	152	76
D201	98	73
D202	90	73

The maximum capacity of resin (q_m) is calculated from the equation: $q^* = q_m c/(K_d + c)$; for details, see the "Adsorption Isotherm" section. Recovery (%) = collected CTP/initial CTP.

recovery of 201×7 was a little higher than that of 201×8. 201×7 and 201×4 had different strongly basic ion exchange groups, namely, type I and type II, respectively. The basic strength of type II was slightly weaker than that of type I. The adsorption capacity of type I was a little greater than that of type II. However, there were no significant effects of basic strength of anion exchange resin on the recovery. 201×7 resin had proper pore size and high adsorption capacity with high recovery.

Adsorption Isotherm

The adsorption isotherms obtained at pH 3.0 and 6.0 on resin 201×7 are shown in Fig. 3a, b. The isotherm obtained at pH 6.0 was a Langmuir form. The curve was fitted by Langmuir equation and parameters were calculated. Typical hyperbolic Langmuir isotherm equation (simple, single-site single-sorbate), $q^*=q_m c/(K_d+c)$, where q^* is the amount of solute absorbed in equilibrium, c is the concentration of solute in the bulk solution, q_m is the maximum capacity of resin-saturated solute, and K_d is the dissociation

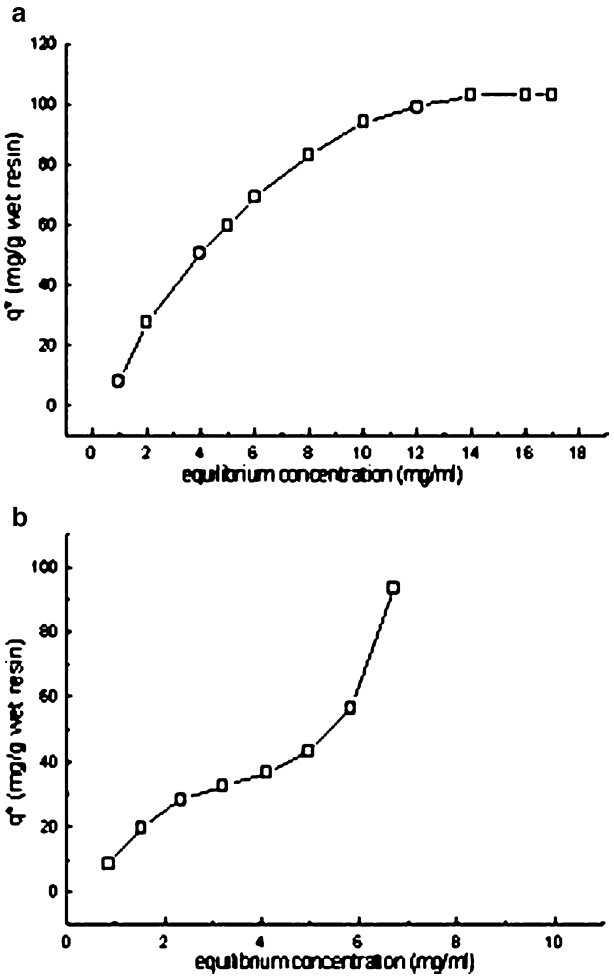

Fig. 3 (**a**) Isotherm of 201_7 anion ion exchange resin absorbing standard cytidine 5′ triphosphate (pH 6.0). (**b**) Isotherm of 201_7 anion ion exchange resin absorbing standard cytidine 5′ triphosphate (pH 3.0)

constant, which was frequently used to describe the adsorption performance of ion exchange [18, 19]. The dissociation constant (K_d) was 12.35 mg/ml and the maximum capacity (q_m) of resin 201×7 was 232.4 mg/g wet resin.

The isotherm shape obtained at pH 3.0 was not a Langmuir isotherm form. It belonged to the type II adsorption. Ruthven [20] divided the adsorption isotherms into five classes. Type II was generally observed only in adsorbents with a wide range of pore size distribution. In such systems, there was a continuous progression of adsorption, with the increase in loading from monolayer to multilayer adsorption [21]. The type II isotherm showed that interaction between 201×7 and CTP was not ion exchange interaction, but the results of complex interactions including physical and chemical mechanisms, such as acid–base interaction, hydrogen bond interaction, and hydrophobic interaction [22].

Selection of Elution and Washing Conditions

The elution results of various eluants for CTP absorbed on 201×7 at pH 6.0 were shown in Table 3. The recovery of CTP increased with the increase of NaCl concentration. The highest elution recovery of 98.8% was obtained with 2.0 M NaCl. However, 1.0 M NaCl also showed a rather high-elution recovery of 96.9%. As there was not much difference in the elution of CTP between 1.0 M NaCl and 2.0 M NaCl, 1.0 M NaCl was used for the elution of CTP in later column separation. The elution of CTP by NaCl was found to be more effective than others.

As chloride was a monovalent anion, probably it had stronger binding ability to resin than divalent anion did such as SO_4^{2-}. Mixture of methanol and NaCl did not show good performance in the elution of CTP. It seemed that methanol decreased NaCl elution effect. Methanol was an organic solvent with low dielectric constant and this probably reduced the elution effect of NaCl. However, methanol was a good eluant for lactic acid adsorbed on VI-15, a weak basic anion exchange resin [23]. The elution recovery of CTP from 201×7 using 20% (*v*/*v*) methanol was only 19.36%. Ammonia or mixture of ammonia and methanol showed medium elution ability. Elution recovery of CTP was enhanced at higher concentration of ammonia. However, elution recovery of ammonia was always lower than that of NaCl.

Table 3 Elution of CTP adsorbed on 201×7 at pH 7.0 by various eluants.

Elution agents	Concentration in eluate (mg/ml)	Recovery (%)
0.5 M NaCl	2.12	80.56
1.0 M NaCl	2.55	96.90
1.5 M NaCl	2.59	98.42
2.0 M NaCl	2.60	98.80
20% (*v*/*v*) methanol	0.51	19.36
50% (*v*/*v*) methanol	0.34	12.92
70% (*v*/*v*) methanol	0.27	10.26
0.5 M $(NH_4)_2SO_4$	1.23	46.74
1.0 M $(NH_4)_2SO_4$	1.57	59.66
1.5 M $(NH_4)_2SO_4$	1.63	61.94
2.0 M $(NH_4)_2SO_4$	1.64	62.32

Loaded sample was only standard CTP. Recovery (%) = collected CTP/loaded CTP; 201×7 was a kind of strong anion ion exchange resin.

CTP = cytidine 5′-triphosphate.

Table 4 Elution of CTP adsorbed on 201×7 at pH 6.0 by various concentration eluants.

Elution manners	Part 1		Part 2	
	CMP (%)	CTP (%)	CMP (%)	CTP (%)
0.1–1.0 M NaCl	100	0	10	90
0.2–1.0 M NaCl	100	0	7.0	93
0.3–1.0 M NaCl	98	1.0	3.0	97
0.4–1.0 M NaCl	97	3.0	0	100
0.5–1.0 M NaCl	85	15	0	100
0.6–1.0 M NaCl	73	27	0	100
0.7–1.0 M NaCl	54	46	0	100

Loaded sample was mixture of standard CMP and CTP. The elution consisted of two steps. The first was elution by an initial concentration of NaCl. Then it was eluted by NaCl at a constant concentration of 1.0 M.

As we can see, recovery of CTP could be obtained the highest when NaCl is used as eluant. Using one concentration of NaCl, CMP, and CTP could not be separated entirely. Different elution manners were studied to separate CTP from CMP. All the experiments were carried out in a stepwise manner. The elution consisted of two stages where an increasing stepwise gradient of sodium chloride was used (0.1 and 1.0, 0.2 and 1.0, 0.3 and 1.0, 0.4 and 1.0, 0.5 and 1.0, 0.6 and 1.0, 0.7 and 1.0 M). The results of various elution manners are shown in Table 4. The elution curve in ion exchange chromatography using 0.4–1.0 M NaCl is shown in Fig. 4. And the graphs of other elution manners had the similar profiles with this.

Purified CTP could be collected mainly in peak 2 (part 2). It was obvious that more and more CTP was lost in peak 1 with the increase of initial NaCl concentration. When the initial NaCl concentration reached 0.4 M, all the CMP was eluted in the first peak and the substance in the second peak was totally CTP. When the initial NaCl concentration was from 0.4 to 0.7 M, peak 2 was thoroughly CTP. The loss of CTP in the first peak was the least when the initial NaCl concentration was 0.4 M. When the initial elution concentration was from 0.1 to 0.3 M, some CMP existed in peak 2. Therefore, 0.4 M NaCl was selected as the initial elution concentration, followed by 1.0 M NaCl.

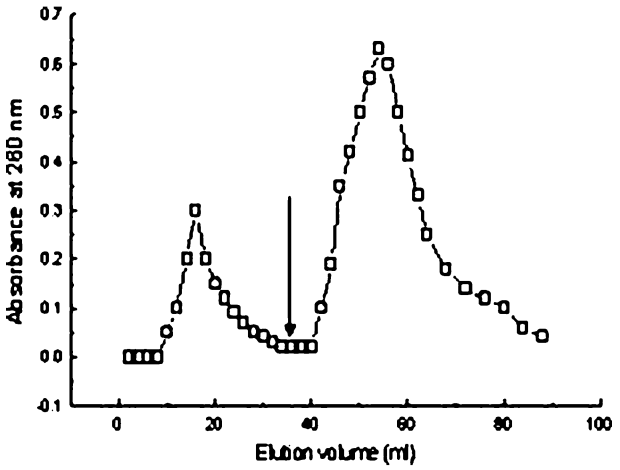

Fig. 4 Elution curve of standard mixture of CMP and CTP, absorbed on 201_7 strong anion exchange resin at pH 7.0 using a stepwise elution manner: From beginning to the *arrowhead* the column was eluted with 0.4 M NaCl; from the *arrowhead* to the end the column was eluted with 1.0 M NaCl

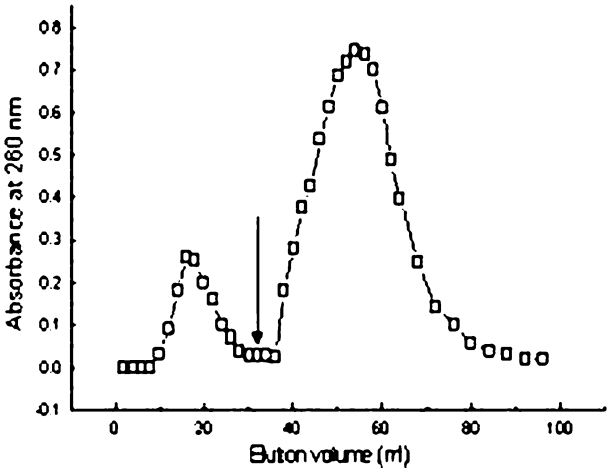

Fig. 5 Elution curve of standard mixture of CMP and CTP, absorbed on 201_7 strong anion ion exchange resin using a step-wise elution manner: From beginning to the *arrowhead* the column was eluted with 0.4 M NaCI; from the *arrowhead* to the end the column was eluted with 1.0 M NaCI. The flow rate was always 0.8 ml/min

The effects of different flow rates (0.2, 0.5, 0.8, 1.0, 1.2, 1.5, 1.8, and 2.0 ml/min) on the separation efficiency of CTP were investigated on a 10/30 column. Ten-gram 201×7 resin was packed into the column with a bed height of 22.5 cm (CV: 19.8 ml). Then, 5-ml cytidine compounds (about 25 mg/ml, pH 6.0), 20-ml distilled water was in turn pumped into the column in the downward direction. After that, the column was initially eluted by 0.4 M NaCl. When the absorbance at 260 nm was lower than 0.005, the column was eluted by 1.0 M NaCl. Finally, all the solutions through the column were collected for HPLC analysis. Other operational procedures were the same as described in the "Isolation of CTP by Ion-Exchange Resins" section. The elution curve at flow rate of 0.8 ml/min in ion exchange chromatography is shown in Fig. 5. The graphs of other flow rates had a similar profile to this.

Purified CTP could be collected in peak 2 easily because 0.40 and 1.0 M NaCl were weaker and stronger eluants to CTP, respectively. Table 5 gave the results of CMP and CTP purification under different flow rates of 0.2, 0.5, 0.8, 1.0, 1.2, 1.5, 1.8, and 2.0 ml/min. It was evident that the recovery yield (%) of CTP increased with the decrease of flow rate, which

Table 5 Effect of different flow rates on CTP separation.

Speed (ml/min)	Retention time (min)		Recovery of CTP (%)	Peak 2	
	Peak1	Peak2		CMP (%)	CTP (%)
0.20	50	180	93	10	90
0.50	30	120	92	3.0	97
0.80	18	69	90	1.0	99
1.00	15	60	85	3.0	97
1.20	12	48	82	5.0	95
1.50	10	38	71	8.0	92
1.80	8	30	64	10	90
2.00	7	26	60	15	85

Loaded sample (pH 6.0) was a mixture of standard CMP and CTP. The elution consisted of two steps: first, the column was eluted with 0.4 M NaCl. When the absorbance at 260 nm was lower than 0.005, the column was eluted with 1 M NaCl. Recovery of CTP (%) = collected CTP / loaded CTP.

CMP = cytidine 5′-monophosphate; CTP = cytidine 5′-triphosphate.

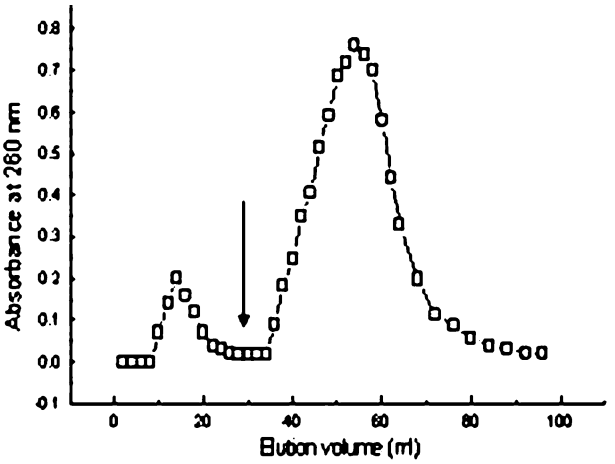

Fig. 6 Elution curve of standard mixture of CMP and CTP, absorbed on 201_7 strong anion exchange resin at pH 6.0 using a stepwise elution manner: From beginning to the *arrowhead* the column was eluted with 0.4 M NaCI; from the *arrowhead* to the end the column was eluted with 1.0 M NaCI. The flow rate was always 0.8 ml/min. The ratio between sample volume and resin loaded was 0.5 ml/g

was imaginably attributed to the different diffusion rates of solutes in CMP and CTP mixture [24]. At the same time, when the flow rate was above 0.80 ml/min, the content of CTP in peak 2 was obviously decreased. However, the retention time in parts 1 and 2 were decreased with the increase of flow rate. Among all, 0.8 ml/min was the most suitable flow rate.

The effects of different ratios between sample volume loaded and resin 201×7 loaded (0.1, 0.5, and 1.0 ml/g) on the separation efficiency of CMP and CTP were investigated on a 10/30 column. Ten-gram 201×7 resin was packed into the column to a bed height of 22.5 cm (CV: 19.8 ml). pH and flow rate in this investigation were 6.0 and 0.8 ml/min, respectively. The regeneration/transformation procedure of resin 201×7 was the same as 2.4. The separation graph of CTP at the ratio of 0.5 is shown in Fig. 6. The profiles in other ratios were similar to this.

The details obtained about sample volume loaded are given in Table 6. The recovery yield (%) of CTP in part 2 was increased (from 83.9 to 94.3%) with the decrease of the ratio (from 1.0 to 0.1). Particularly, CTP (%; from 95.3 to 99.2%) ascended with the decrease of the ratio (from 1.0 to 0.1), too. After analysis, we found the amount of CMP decreased with the increase of sample loaded, but the amount of CTP increased with the decrease of sample volume loaded. This was, perhaps, because of the stronger affinity between CTP with resin 201×7 compared with that of CMP with the resin. Distinctly, a lower ratio could improve the purity and yield, but reduced the productivity during CTP purification process. Therefore, the ratio 0.5 (ml/g) between the sample volume and the resin loaded was chosen.

Table 6 Effect of sample volume loaded on resin separation.

Ratio	Part 1		Part 2		Recovery of CTP (%)
	CMP (%)	CTP (%)	CMP (%)	CTP (%)	
0.1	97.8	2.2	0.8	99.2	94.3
0.5	94.4	5.6	1.5	98.5	90.5
1.0	90.9	9.1	4.7	95.3	83.9

Ratio (ml/g) = sample volumes loaded (ml) /resin loaded (g). Loaded sample was a mixture of standard CMP and CTP. The elution consisted of two steps: first, the column was eluted with 0.4 M NaCl. When the absorbance at 260 nm was lower than 0.005, the column was eluted with 1 M NaCl. Recovery of CTP (%) = collected CTP/loaded CTP.

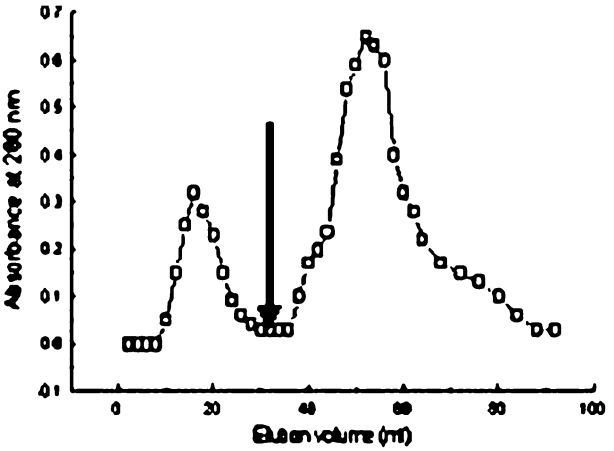

Fig. 7 Elution curve of reaction product, absorbed on 201_7 strong anion exchange resin at pH 6.0 using a stepwise elution manner: From beginning to the *arrowhead* the column was eluted with 0.4 M NaCI; from the *arrowhead* to the end the column was eluted with 1.0 M NaCI. The flow rate was always 0.8 ml/min. The ratio between sample volume and resin loaded was 0.5 ml/g

Separation of reaction products is shown in Fig. 7. Purity and recovery of CTP were 95% and 96%, respectively. This method was suitable for the purification of CTP reaction products. It could get a good peak shape with a high recovery and purity.

Liquid Chromatography Method for Analysis of Cytidine Compounds

The analysis of cytidine compounds in biological samples is of great practical importance. A limitation of current HPLC techniques is still the time required for the individual HPLC run, which is in the order of 15–60 min. Furthermore, no literature has been aimed at separating cytidine compounds. The present report permits to shorten the run time to less than 7 min with excellent peak resolution and compound sensitivity. All the compounds were identified by comparing their retention time with that of a standard compound. High-performance liquid chromatography was carried out on a Shim-pack WAX-1 column (50× 4 mm, 5 um, Shimadzu, Japan) using UV detector at 260 nm at a flow rate of 1 ml/min. The mobile phase was 480 mM potassium dihydrogen phosphate solution (pH 5, adjusted with 100 mM NaOH). These conditions were the optimal of one-factor-at-a-time (constituting mobile phase, concentration, and pH of the mobile phase, flow rate, injection volume; data not shown), which represented a good compromise between good separation and high recovery. The linearity of the method was proven in standards at 10 known concentrations. Calibration curves were obtained by unweighted least-squares linear regression analysis of the peak area of the respective cytidine compounds (CMP, CDP, CTP). The method gave

Table 7 Correlation coefficients of calibration curves and detection limits.

Component	r^2 value	Regression	Detection limit (μg/ml)
CMP	0.9989	A=18751C−18634	20–600
CDP	0.9980	A=23053C+16438	20–800
CTP	0.9983	A=47870C+23679	200–5,000

Each regression and correlation coefficient was calculated from ten measurements. Injection volume was 10 μl. A: peak area; C: concentration of each nucleotide (μg/ml). HPLC was carried out on a WAX-1 column using a UV detector at 260 nm at a flow rate of 1 ml/min. The mobile phase was a 480 mM potassium dihydrogen phosphate solution (pH 5, adjusted with 100 mM NaOH). Data are the mean of three different preparations.

Table 8 Recovery of CMP, CDP and CTP.

Component	CMP	CDP	CTP
Nominal amount (μg)	60.40	60.40	120.00
Experimental amount (μg)	60.13	59.97	118.23
Recovery (%)	99.55	99.28	98.53

All the injection volume was 10 μl at a flow rate of 1.0 ml/min. Data are the mean of three different preparations.

linear relationships between the signal intensity recorded and the amount of compound analyzed in different ranges. The r^2 values for three independent determinations were between 0.998 and 0.999. There was no significant difference of the correlation coefficients (Table 7) when evaluating peak area or peak height as measures of signal intensity.

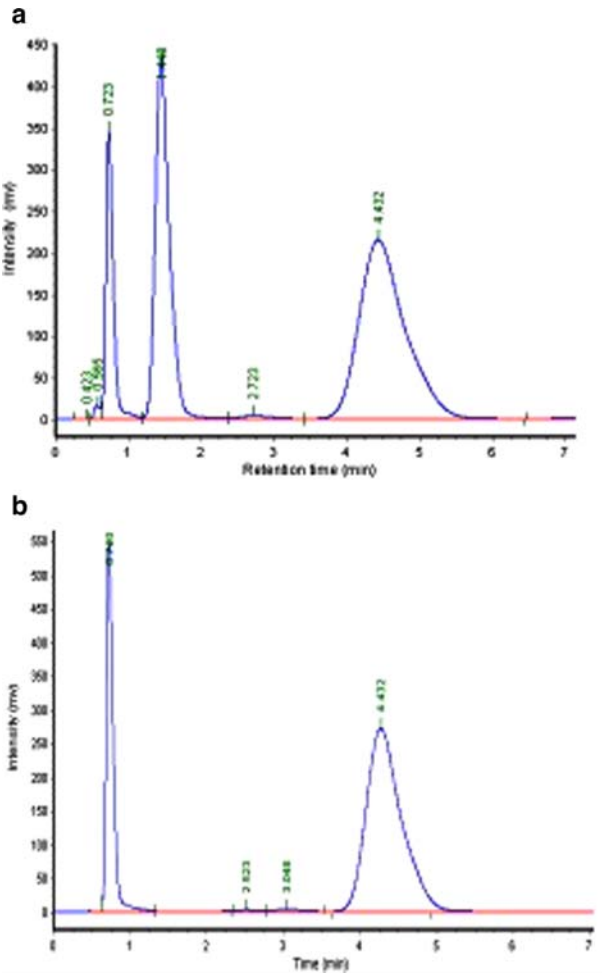

Fig. 8 (**a**) Chromatograph of mixture of standard CMP, CDP, CTP using detection at 260 nM. The retention time for CMP, CDP, CTP, were 0.723, 1.448, 4.432 min, respectively. (**b**) Chromatograph of reaction products using detection at 260 nM

To assess the accuracy of the method, a selected extract cytidine compound was spiked with standard cytidine compound at known concentrations. The ratio of the average measured concentration and its nominal concentration was defined as the recovery. Results are shown in Table 8. Each compound had a good recovery and all the recoveries were higher than 98%.

Reproducibility of HPLC analysis was determined by repeated injection of standard solutions containing three nucleotides in various concentrations ranged from 200 to 600 μg/ml. The results obtained in a period of 2 months showed a relative standard deviation (RSD) in the range from 2 to 4%. The chromatograms resulting from the injection of pure standards and reaction products by biosynthesis under the chromatographic conditions finally adopted were presented in Fig. 8a, b. The retention time for CMP, CDP, and CTP were 0.723, 1.448, and 4.432 min, respectively. From the difference between the two figures, we could conclude that CDP did not exist in the biosynthesis process. It was possible that all the CDP produced was thoroughly converted to CTP.

This method is extremely credible. The present method also reduces the necessary analysis time considerably and thereby makes the use of HPLC more efficiently. In conclusion, we have evaluated a new rapid HPLC method for the analysis of cytidine compounds in biological samples with high precision and repeatability.

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