Indian Journal of Chemistry
Vol. 63, March 2024, pp. 304-314
DOI: 10.56042/ijc.v63i3.6700

Synthesis of process related impurities of Citicoline and Citicoline sodium[#]

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Received 11 November 2023; accepted (revised) 26 February 2024

Citicoline is the diphosphate ester of cytidine. The said compound is sensitive to chemical atmosphere and exhibits formation of process/degradation impurities. Herein is reported a comprehensive synthesis of the pharmacopeia impurities of Citicoline and Citicoline sodium with an objective to provide impurities for better understanding and control in the Citicoline preparation process. This report provides synthetic methodologies for the impurities, considering ease of handling, chemistry and eliminating tedious purification. All the impurities are characterized and the structures confirmed.

Keywords: Citicoline, Citicoline sodium, Cytidine, Cytidine-5′-monophosphate, Impurities

Citicoline (Cytidine 5′-diphosphocholine)/Citicoline sodium is a drug used for potential neuroprotective and nootropic activity. CDP-choline (citicoline) is composed of ribose, pyrophosphate, cytosine (a nitrogenous base), and choline[1] (Fig. 1). The synthesis of the Citicoline is either by synthetic methdology[2-8] or enzymatic pathway[9,10]. Honjo and Furukawa reported synthesis of Citicoline from Cytidine-5′-monophosphate, wherein Cytidine-5′-monophosphate was reacted with 4-morpholino-N,N′-dicyclohexylcarbodiimide followed by reaction with chloride salt of choline phosphormorpholidate[2].

Process related/degradation impurities are integral part of process development of drug substances. It is essential to know the origin and method of control of any unwanted substances (impurities) present in it. The limit should be controlled under the threshold of toxicological concern (TTC) for the purpose of ensuring safety and efficacy of the drug and to meet the requirements of various drug regulatory agencies[11,12]. There are numerus reports for the preparation of Citicoline but there are only couple of references disclosing synthesis and isolation of the process related/degradation impurities[15,16,19]. The impurities in drug substances mostly come from starting substrates, reagents, solvents and side reactions of the synthetic route employed. Therefore, assessment and control of the undesired substances is an essential aspect of the drug development journey, with special consideration of patient health risk[13,14].

For Citicoline and its sodium salt, Pharmacopeia (USP, IP) discloses the related impurities. To our best knowledge, there is only couple of literature reports defining either synthesis or isolation of some of impurities. There is not a single report confirming structure for all disclosed impurities[15,16,19]. Goa *et al.*[15] discloses preparation of uridine diphosphocholine impurity and shown the HPLC-mass correlation for the said impurity. Even though the impurity is disclosed its isolation and spectral characterization confirming the structure of the impurity is not included in the said paper. Further to this Bergmeyer *et al.*[16], reports synthesis of Cytidine diphosphate ethanolamine (herein as Cytidine 5′-diphosphoethanolamine) but is silent for purity of the desired compound and its confirmation for identity. Synthesis of Uridine diphosphocholine is even though disclosed by Sun *et al.*[19], the synthetic methodology is cumbersome to workout in lab. There are seven impurities enlisted in pharmacopeia (USP and IP) for citicoline, out of which one is cytidine-5′-monophsophate which is taken as starting material for synthesis of Citicoline. Whereas Cytidine 5′-dihydrogen phosphate is not derived *via* synthesis protocol. Herein we report simple synthetic procedures and characterization of listed impurities of Citicoline/ Citicoline sodium from US and Indian pharmacopeia excluding two impurities as Cytidine-5′-monophosphate (starting material for Citicoline) and Cytidine-5′-dihydrogenphosphate (impurity in starting material) in order to evaluate their origin/fate during drug substance development. The said impurities are enlisted in Table 1.

Standards Availability: Standards of the impurities are available from the authors.

We desired to develop a commercially viable and robust process for producing both Citicoline and its sodium salt as drug substance(s), while conforming to drug regulatory requirements. In this process, we observed that there are many synthetic protocols reported in the prior art literature for the preparation of Citicoline (**I**) and its sodium salt[2-10]. However, most of the synthetic routes were found to have use of Cytidine 5′-monophosphate (**II**) as the key starting material, excluding[5-10].

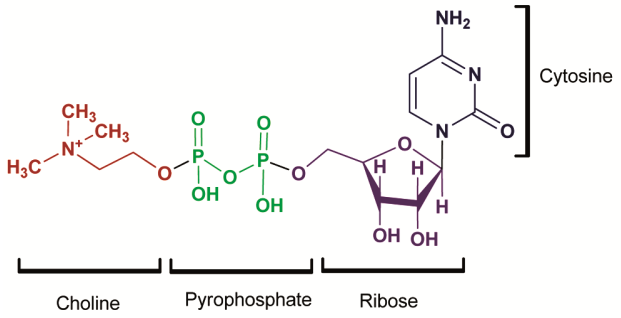
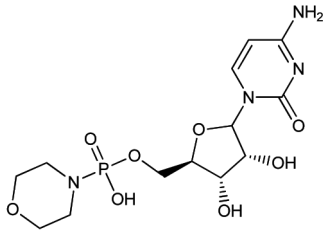
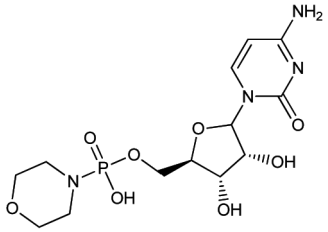
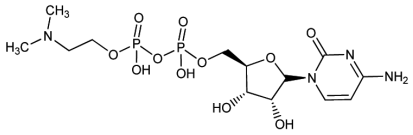
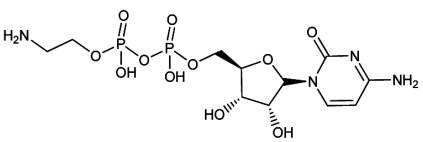
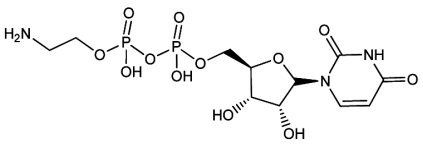


Fig. 1 — Citicoline (I)

The synthetic pathway reported by Kumar *et al*.[7] attracted us to explore synthesis of Citicoline. The said process involves preparation of Cytidine-5′-monophosphomorpholidate (**III**), and in next stage it is reacted with calcium phosphorous choline chloride to give Citicoline.

Experiments in line with the patent application[7] were performed and the results obtained at a crude-Citicoline were showing presence of two major impurities. HPLC chromatogram shows one being Cytidine-5′-monophosphate (**II**), whereas second

Table 1 — Impurities of Citicoline/ Citicoline sodium

S. No.	Impurity Name	Structure	Source of Impurity	Listed in Pharmacopeia
1	Cytidine-monophospho morpholidate		In-process transition	Citicoline sodium (IP)
2	Cytidine-5′-monophosphate methyl ester		In-process impurity	Citicoline sodium (USP); Citicoline sodium (IP)
3	Desmethyl cytidine-5′-diphsophocholine sodium		From starting material	Citicoline sodium (IP)
4	Cytidine 5′-diphosphoethanolamine		From Starting material	Citicoline (USP)
5	Uridine 5′-diphosphocholine		Process related impurity	Citicoline (USP)

major impurity was Cytidine-5′-monophosphate methyl ester (**IV**). These both impurities are listed for Citicoline sodium in US pharmacopeia and Indian pharmacopeia. Both impurities even though looks very simple but are crucial for process development of Citicoline sodium.

Desmethylciticoline (**V**) is an impurity reported in Indian pharmacopeia. The synthetic path way for disclosed impurity (**V**) is not reported in the literature.

Cytidine 5′-diphosphoethanolamine (**VI**) is an impurity enlisted in USP for Citicoline. The potential risk for impurity is due to presence of calcium phosphoryl ethanol amine hydrochloride (**1**) in calcium phosphoryl choline chloride (**2**). Even though synthesis of said impurity is reported by Ghezal *et al*.[20] The disclosed synthesis involves activation of cytidine-5′-monophosphate by making imidazole phosphate derivative followed by reaction with N-Boc-2-aminoethylphosphate in presence of zinc chloride in anhydrous condition followed by deprotection of N-carbamate using TFA and purification by column chromatography. Summarized procedure is very tedious and with our hands we are unable to get desired purity.

Additionally Uridine diphosphocholine (**VII**), is listed impurity in Citicoline USP. It is observed to be formed during acidic environment of reaction or workup procedure.

Thus, the previous research works inspired us toward reporting comprehensive report thereby deriving systematic synthesis and confirmation of structures using spectral methods.

Experimental Section

Cytidine-5′-monophosphate was purchased from Meteoric Biopharmaceutical Pvt Ltd. and sodium salt of uridine-5′-monophosphate was purchased from SRL. FT-IR spectra of samples were recorded on Jasco-FTIR-4200 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Agilent NMR VNMRS 400 spectrometer using tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (δ), coupling constants (*J*-values) are reported in Hertz (Hz), and spin multiplicities are indicated by the symbols as: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), brs (broad singlet). The experiments to determine the molecular mass were performed on a G6160 A Infinity Lab LC/MSD/IQ mass spectro-photometer from Agilent (Singapore) equipped with electrospray ionization (ESI) source.

Synthesis of Cytidine 5′-monophosphomorpholidate, III

Cytidine 5′-monophosphate (10g, 30.94 mmol) was dissolved in methanol (60 mL) and subsequently morpholine (8.76 g. 100.5 mmol) was added. DCC (12.76 g, 61.8 mmol) in methanol (20mL) was added slowly to the above solution[17]. The mixture was heated to 50-55 °C and kept at this temperature for 12 hours. After the completion of reaction as monitored by HPLC[28], reaction mass was cool to 15-20°C and filtered off. Mother liquor was concentrated under reduced pressure. Obtained residue was dissolved in water (10mL) and filtered off as to remove presence of traces of DCU. Aqueous layer was lyophilized to get desired compound. Product was further purified from ethyl acetate to give desired product (11g, 90.61% Molar yield) as white solid. HPLC[28]: 98.43%; FTIR (KBr): 3190, 2929, 2855, 1648, 1610 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.204- 1.300 (m, 4H, 2×CH_2), 1.343-1.427 (m, 4H, 2×CH_2),1.558-1.590 (brs, 2H, CH_2), 1.705-1.737 (d, 4H, CH_2), 1.786-1.813 (d, 4H, 2×CH_2), 1.89 (s, 1H, NH of carbodimide), 2.892-2.903 (q, 4H, 2×CH_2), 3.225 (brs, 2H, CH_2), 3.356 (t, 4H, 2×CH_2), 3.440-3.4460(t, 4H, 2×CH_2), 3.626-3.637 (m, 4H, 2×CH, NH_2), 3.803-4.055(m, 6H, 3×CH_2), 5.423 (brs, 1H, CH), 5.711-5.729 (d, 1H, CH, J = 7.2 Hz), 5.597-5.787 (d, 1H, ArH, J = 7.6Hz), 7.031 (brs, 1H, OH), 7.344 (brs, 1H, OH), 7.809(brs, 1H, OH), 7.98(d, 1H, ArH, J =7.6Hz); ^{13}C NMR (100MHz, DMSO-d_6): δ 165.576, 155.556, 141.163, 94.150, 88.601, 83.021, 82.945, 74.526, 70.078, 67.307, 66.920, 66.851, 65.819, 63.322, 53.939, 48.572, 48.116, 46.029, 45.535, 33.351, 32.812, 25.342, 24.811; MS (ESI): *m/z* $[M+H]^+$ Calcd for $C_{13}H_{21}N_4O_8P$: 392.3. Found: 392.3.

Synthesis of Cytidine 5′-monophosphate methyl ester, IV

Cytidine 5′-monophosphate (10g, 15.47 mmol) was dissolved in methanol (60 mL). To the resulting solution DCC (9.56 g, 46.33 mmol) was added slowly, the mixture was stir at 30-35 °C for 20 hrs. Precipitated DCU was filtered off. Mother liquor was concentrated under reduced pressure at 30-35°C, traces of solvent was removed by drying under high vacuum. Obtained residue was stir in water (10mL). Aqueous layer was filtered off, to remove DCU. Aqueous solution was extracted with tertiary butanol (2×5mL) and aqueous layer concentrated under vacuum at 50-55°C and was purified by column chromatography through silica (60-80 mesh) and

crystallisation from Methanol: Acetone (1:1) to give desired product as white solid (5.0 g, 47.3% molar yield). HPLC purity[28]: 98.82; FTIR (KBr): 3179, 2934, 2857, 1724, 1650, 1581. 1492 cm^{-1}; ^1H NMR (400 MHz, D_2O): δ 7.912-7.893 (d, 1H, ArH, J = 7.6 Hz), 6.043-6.062 (1H, ArH, J = 7.6 Hz), 5.935-5.944 (d, 1H, CH, J = 3.6Hz), 4.245-4.293 (q, 2H, CH_2), 4.232-4.207 (m, 1H, CH), 4.169-4.122(m, 1H, CH), 4.022-4.064(m, 1H, CH), 3.575 (d, 3H, CH_3, $J_{H,P}$ = 10.8 Hz); ^{13}C NMR (100 MHz, D_2O): δ 165.837, 157.330, 141.136, 96.241, 89.336, 82.392 ($J_{C,P}$ = 33.6Hz), 74.022, 69.074, 64.050($J_{C,P}$ = 18.4 Hz), 52.811 ($J_{C,P}$ = 24.4 Hz); MS (ESI): *m/z* $[M+H]^+$ Calcd for $C_{10}H_{16}N_3O_8P$: 336.2. Found: 336.6.

Synthesis of Desmethyl Cytidine-5′-diphosphocoline, V

Cytidine 5′-monophosphate (5g, 15.47 mmol) was dissolved in methanol (30 mL) and subsequently morpholine (4.38 g. 50.28 mmol) was added. DCC (6.38 g, 30.92 mmol) was added slowly[17], the mixture was heated to 50-55 °C and kept at this temperature for 12 hrs. Reaction was monitored for complete conversion by HPLC[28]. After completion of reaction, reaction mass was cool to 15-20°C. Filter off reaction mass to remove DCU and obtained mother liquor was concentrated under vacuum. Crude Cytidine-5′-monophosphomorpholidate (**III**) (in the residue form) was dissolved in 2-methoxy ethanol (25mL). Desmethylphosphorylcholine chloride (Compound **4**) (7.75 g, 46.37 mmol) was dissolved in 2-methoxy ethanol (40 mL) and heated to 50-55 °C. Solution of compound (**III**) was added to desmethyl-phosphorylcholine chloride solution, maintaining *p*H of reaction mass in range of 3.5 to 4.0 using 10% Methanolic HCl solution. Reaction mass was stirred for 4 hours at 50-55°C. Reaction mass was filtered off and the solid residue was dissolved in water (10mL). The said crude as aqueous solution was purified by column containing strong basic anion exchange resin (Dowex 1×2 in formate form; 50-100 mesh), The fraction with HPLC purity ≥ 97% were concentrated and crystallisation from Acetone to give desired product (1.85g, 25.24% Molar yield) as off white solid. HPLC[28]: 97.38%; FTIR (KBr): 3313, 2363, 1721, 1675, 1540, 1486 cm^{-1}; ^1H NMR (400 MHz, D_2O): δ 8.168-8.188 (d, 1H, ArH, J = 8Hz), 6.283-6.302 (d, 1H, ArH, J = 7.6 Hz), 5.946-5.953 (d, 1H, CH, J = 2.8 Hz), 4.211-4.356 (m, 8H), 3.447-3.471 (t, 2H, N-CH_2, J = 4.6Hz), 2.954-2.963 (s, 6H, 2×CH_3); ^{13}C NMR (100 MHz, D_2O): δ 159.724, 149.240, 143.668, 95.266, 89.511, 83.059($J_{C,P}$ = 9.1 Hz), 74.192, 69.007, 64.445, 59.822 ($J_{C,P}$ = 3.6Hz), 57.233 ($J_{C,P}$ = 7.6Hz), 42.741; MS (ESI): *m/z* $[M+Na]^+$ Calcd for $C_{13}H_{24}N_4O_{11}P_2$: 496.279. Found: 495.8.

Synthesis of Desmethyl Cytidine- 5′-diphosphocoline (V) from N,N-dimethyl ethanol, 5

Synthesis of the said impurity was carried out with paralled methodology and the details process is enlisted herein,

Preparation of N-benzyl-N,N-dimethyl aminoethanol hydrochloride, 6

The said conversion is carried out with reference to procedure disclosed by Wurm *et al*.[21] Benzyl chloride (29.82g, 235.6mmol) was slowly added to the solution of N,N-Dimethylaminoethanol (20g, 224.4mmol) in toluene (100mL) at 50°C. Stir for 2 hrs and gummy stuff was precipitated, after completion of reaction, acetone (100mL) was added to precipitate the product, which turns oily. Isolated product 45g with 78% yield. The said material was used as such for next stage.

Preparation of calcium salt of N-benzyl-N,N-dimethyl-2-phosphonoethanaminium chloride, 7

Synthesis of titled compound was carried out using compound (**6**) and phosphorous oxychloride in 2-dichlorobenzene as reported[22]. The said product was obtained as white solid. % Molar Yield: 56%. Melting point: >240°C (Decomposition). Calcium content: Observed: 12.6%w/w (theoretical: 11.98% w/w), Chloride content: Observed 11.05% w/w (theoretical: 10.63%w/w). The said material was used without further purification for next step.

Preparation of N-Benzyl-N,N-dimethyl Cytidine-5′-diphosphoethanolamine, 8

Compound (**7**) (15.66g, 38.67 mmol) was taken in methanol (80mL). Cytidine-5′-monophospho-morpholidate (**II**, 5g, 15.4 mmol) was added adjusting reaction mass *p*H at 3.5-4 using methanolic hydrochloric acid. During the addition of cytidine-5′-monophosphomorpholidate temperature of reaction mass was maintained at 50-55°C. Stir for additional 2.5 hours at 50-55°C. After completion of reaction as monitored by TLC, precipitation of crude product was carried out using isopropyl alcohol (20mL) and water (5 mL). Filtered off solid and dried under vacuum to give 7.5g crude title product which was used without further purification for next step. % Molar yield: 71%. Melting point:

> 178°C (Decomposition). IR (KBr): 3327, 1721, 1648, 1488, 1249, 1102 cm^{-1}.

Preparation of Desmethyl Cytidine- 5′-diphosphocoline calcium, 9

N-Benzyl-N, N-dimethyl Cytidine- 5′-diphosphoethanolamine (**8**) (7g, 10.95 mmol) was dissolved in 70mL water, *p*H of the resulting solution was maintained at 3.5 using methanolic Hydrochloric acid. 10% w/w Pd/C (0.7g) was added and debenzylation was carried out at 5kg hydrogen pressure at 70°C. Progress of the reaction was monitored by TLC. After completion of reaction, reaction mass was filtered and concentrated under vacuum to afford desired compound (5.5g, 98% Molar Yield). HPLC[28]: 89.81%. The said crude compound was taken further without further purification.

Desmethyl Cytidine-5′-diphosphocoline sodium, V

Compound of formula (**9**) (7g, 3.66 mmol) was dissolved in water (21mL). To the resulting solution, diisopropylamine oxalate (1.41g, 7.37mmol) was added and stir for 30 minutes. Filtered off to remove calcium oxalate. Aqueous layer was stir with 5% Norit carbon (*p*H ~9) and filtered off. *p*H of aqueous layer was adjusted with 20%w/v sodium hydroxide solution. Desired product was precipitated using ethanol (42mL), followed by filtration to afford white solid (3.4g, 52.4% Molar Yield). HPLC[28]: 97.76%. FTIR (KBr): 3316, 2363, 1722, 1675, 1538 cm^{-1}; ^{1}H NMR (400 MHz, D_2O): δ 8.168-8.189 (d, 1H, ArH, J = 8.4Hz), 6.283-6.303 (d, 1H, ArH, J = 8 Hz), 5.946-5.953 (d, 1H, CH, J = 2.8 Hz), 4.211-4.356 (m, 8H), 3.445-3.472 (t, 2H, N-CH_2, J = 4.5Hz), 2.954-2.963 (s, 6H, 2×CH_3); ^{13}C NMR (100 MHz, D_2O): δ 159.741, 149.138, 143.502, 95.305, 89.523, 83.126($J_{C,P}$ = 9.1 Hz), 74.192, 69.018, 64.384, 60.021($J_{C,P}$ = 3.6Hz), 57.149 ($J_{C,P}$ = 7.6Hz), 42.536; MS (ESI): *m/z* $[M+Na]^+$ Calcd for $C_{13}H_{24}N_4O_{11}P_2$: 496.279. Found: 495.8.

Synthesis of Cytidine- 5′-diphosphoethanolamine, VI

The procedure as disclosed for synthesis of desmethyl Cytidine- 5′-diphosphocoline was used with the change of phosphoryl ethanol amine hydrochloride (Compound **10**) instead of Compound **8** to afford desired product as off white solid (2.1g, 30.81% Molar yield). HPLC[28]:97.35%, IR (KBr): 3397, 2971, 2723, 1671, 1637, 1526, 1456, 1319, 1221, 1076 cm^{-1}; ^{1}H NMR (D_2O): δ 8.183-8.202 (d, 1H, Ar-H, J=7.6Hz), 6.291-6.310 (d, 1H, Ar-H, J = 7.6Hz), 5.931(d, 1H, CH, J=3.6Hz), 4.303-4.351 (m, 3H, of ribose and C*H*(H)-O-P(O)), 4.195-4.207 (m, 3H, CH(*H*)-O-P(O), O-CH_2-ribose part), 3.288-3.310 (t, 2H, N-CH_2, J = 4.4Hz). ^{13}C NMR (DMSO-d_6): δ 166.108, 159.109, 148.443, 143.918, 95.137, 83.127, 74.222, 68.992, 64.407, 62.251, 39.879. MS (ESI): *m/z* $[M+H]^+$ Calcd for $C_{11}H_{18}N_4O_{11}P_2$: 445.236. Found: 445.6.

Synthesis of Uridine- 5′-diphosphocholine sodium, VII

Uridine-5′-monophosphomorpholidate was prepared from Uridine-5′-monophosphate (**VIII**, 5g, 15.42 mmol) according to the procedure disclosed for synthesis of Cytidine-5′-monophosphomorpholidate (example **i**), obtained compound was dissolve in 2-methoxy ethanol (25mL). The phosphoryl choline chloride (10.16 g, 46.26 mmol) was added to 2-methoxy ethanol (50 mL). The resulting mixture was heated to 50-55 °C and above solution of compound **(IX)**, was added maintaining reaction mass *p*H within the range of 3.5 to 4.0 by Methanolic HCl. Stir the reaction mass for 4 hours at 50-55°C. Progress of reaction was monitored by TLC and after completion of reaction, reaction mass was filtered off. Titled compound was further purified by dissolving in water and was purified by column containing weakly anion exchange resin (formate form). Fractions with purity ≥ 95%, were concentrated. Desired product was precipitated from 0.1M sodium hydroxide solution in ethanol to give off white solid (2.6g, 37.78 % molar yield). HPLC[28]: 95%; IR (KBr): 3355, 1687, 1599, 1522, 1476, 1379, 1254, 1094 cm^{-1}; ^{1}H NMR (400 MHz, D_2O): δ 7.935-7.914 (d, 1H, ArH, J = 8.4Hz), 5.991-6.001 (d, 1H, CH, J = 4Hz), 5.948-5.968 (d, 1H, ArH, J = 8Hz), 4.285-4.409 (m, 8H), 3.684-3.704 (t, 2H, CH_2, N-CH_2, J = 4Hz), 3.236 (s, 9H, N-$(CH_3)_3$); ^{13}C NMR (100 MHz, D_2O): δ 167.694, 152.845, 141.412, 102.613, 88.599, 82.936 ($J_{C,P}$ = 8.3Hz), 73.607, 69.538, 65.484, 59.889 ($J_{C,P}$ = 4.6Hz), 53.907 ($J_{C,P}$ = 3.8Hz), 30.192. MS (ESI): *m/z* $[M+H]^+$ Calcd for $C_{14}H_{25}N_3O_{12}P_2$: 489.308. Found: 489.3.

Synthesis of Uridine-5′-diphosphocholine sodium, VII

Citicoline (2g, 0.004085 mol) was dissolved in deionized water (6 mL) and subsequently Sodium Nitrite (1.32 g. 0.01916 mol) was added. The mixture was stirred at 30-35 °C for 5 mins, add drop

wise concentrated Hydrochloric acid (0.448 g, 0.01228mol) was added at 30-35 °C. The mixture was heated to 50-55 °C for 3 hrs. Reaction was monitored for complete conversion by HPLC[28], after completion of reaction, reaction mass was treated with activated carbon (20%w/w) at 50-55°C. *p*H of filtered reaction mass was corrected to 7.0 to 7.5 using 10% aqueous sodium hydroxide solution. The desired product was precipitated from acetone (80 mL), and was further purified product from methanol (20 mL) to afford white solid (1.0 g, 47.6% molar yield), HPLC[28]: 96.14%; IR (KBr): 3352, 1684, 1603, 1522, 1474, 1375, 1253, 1093 cm^{-1}; ^{1}H NMR, ^{13}C NMR are in accordance with synthesis defined in experimental procedure (**vi**). MS (ESI): *m/z* $[M+H]^+$ Calcd for $C_{14}H_{25}N_3O_{12}P_2$: 489.308. Found: 489.3.

Results and Discussion

Based on the literature we defined methodology for synthesis of all listed impurities using classical chemistry tools and provides ease methods for synthesis of impurities.

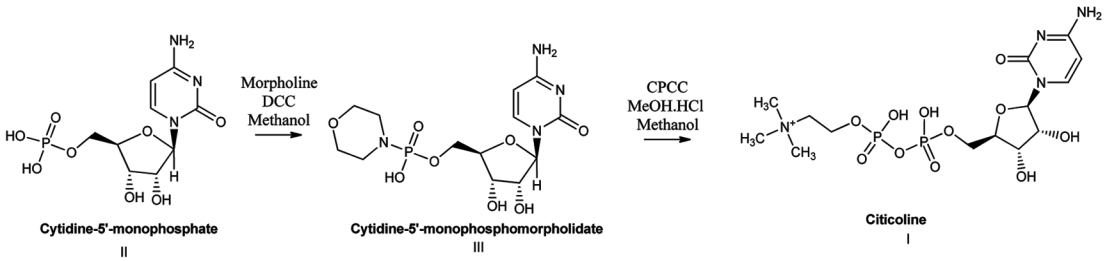
Cytidine 5′-monophosphomorpholidate, III

Cytidine 5′-monophosphomorpholidate **(III)** is a transition as disclosed in Scheme 1, and is listed as an impurity in Citicoline sodium in Indian pharmacopeia. The presence of the impurity may be due to incompletion of reaction. The said impurity was synthesized with respect to the Scheme 1, obtained compound **(III)** shows HPLC purity of ~96% with 2.5% unreacted starting material **(II)**. Because of very labile nature of the targeted impurity, further experimentations were planned as to get pure compound **(III)**, thereby eliminating need for using any purification tools.

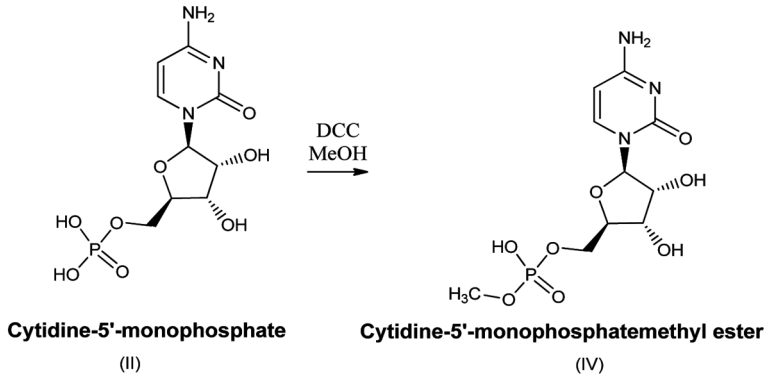
From exhaustive experimentation, process conditions were scrutinized for solvent media and equivalent of DCC and morpholine. 2-methoxy ethanol as solvent and 2.0 equivalent of DCC and 3.25 equivalent of morpholine were conducive for getting 98.43% purity as such with almost absence of starting material **II** (0.08%) and compound **IV** (0.21%). The product obtained was dissolved in water, Filtered off to remove dicyclohexyl urea (DCU). To avoid thermal degradation, aqueous layer was lyophilized to give the desired impurity.

Cytidine-5′-monophsophate methyl ester, IV

Compound **(IV)** is formed due to the reaction of cytidine-5′-monophosphate (**II**) with methanol in presence of Dicyclohexylcarbodiimide (DCC) (Scheme 2). Even though the product formation is quite simple, but biggest challenge to get pure product free



Scheme 1 — Reported procedure for preparation of Citicoline



Scheme 2 — Formation of Cytidine-5′-monophosphatemethyl ester

from cytidine-5′-monophosphate and dicyclohexyl urea (DCU). Considering the labile nature of the desired impurity, experiments were performed to get optimum conversion so as to eliminate presence of cytidine-5′-monophosphate.

With 3.0 equivalent of DCC almost completion of reaction was observed and reaction mass shows HPLC purity ~96% with presence of unreacted compound **(II)** is 3.5% by percentage area normalization. Further screening of process parameters and conditions were carried out but did not improve conversion.

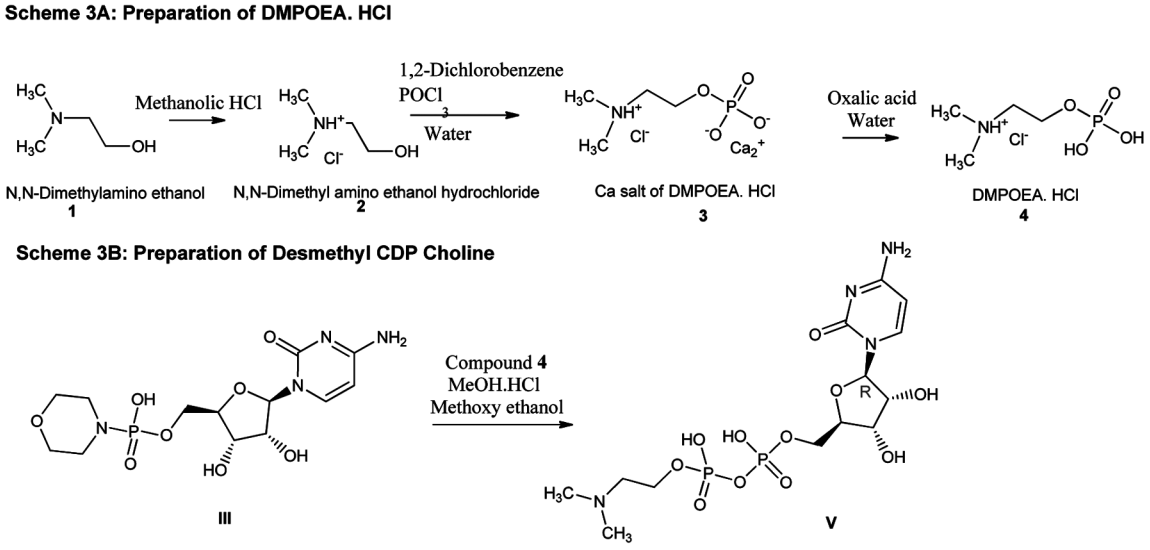
To obtain optimal purity it is required to purify the product, obtained from reaction. Purification of the compound was a challenge as both starting material **(II)** and desired compound **(IV)** are water soluble. Due to the significance of the impurity, it was required to isolate compound (**IV)** free from DCU and starting material **(II)**. Ion exchange column chromatography was employed using strongly basic anion exchange resin with formate as counter ion[16], but the attempt was unsuccessful due to degradation of the product which may be because of the product contains labile ribose and pyrophosphate linkage. The said product was purified by flash chromatography using C18 based stationary phase and water: acetonitrile as solvent for elution, giving no improvement in the product purity. In a later approach the reaction mass was filtered and residue was added to minimum water and filtered off to remove dissolved DCU and extracted with tertiary butanol to remove other nonpolar impurities, resulting aqueous layer was charcolized and concentrated at low temperature. The said compound was purified by column (silica 60-80) chromatography.

Desmethyl cytidine-5′-diphosphocholine sodium (Desmethyl CDP Choline Sodium), V

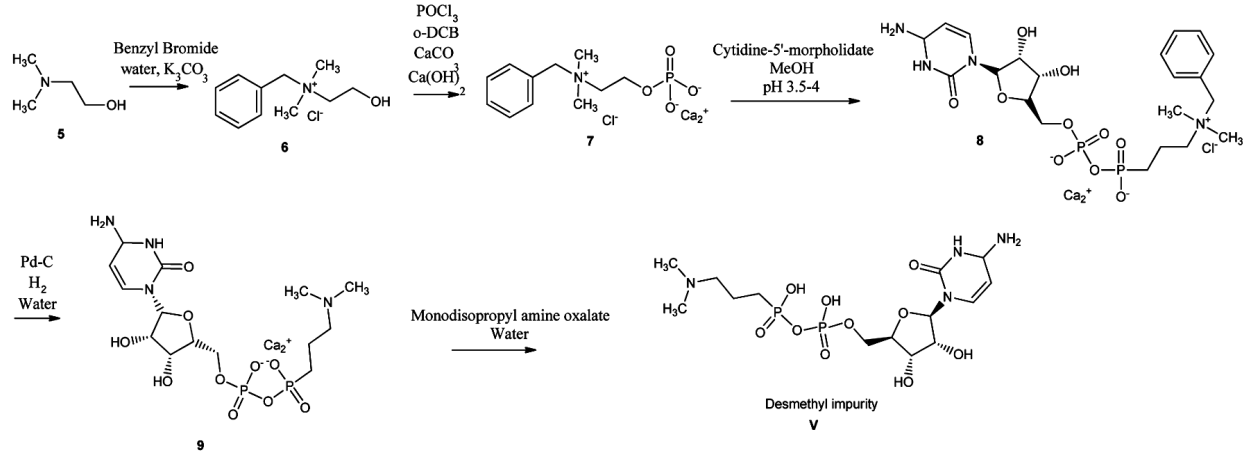
Desmethyl impurity might formed due to the presence of desmethyl choline in calcium phosphorylcholine chloride. The said impurity was synthesized from N, N-dimethyl aminoethanol as illustrated in Scheme 3. Wherein Scheme 3A, summarizes preparation of calcium salt of N,N-dimethyl-2-(phosphonooxy) ethanaminium chloride (Ca DMPOEA.HCl.) from N,N-dimethylamino ethanol hydrochloride in line with the process disclosed for preparation of Calcium choline phosphate[18].

Ca DMPOEA.HCl (**4**) was used for condensation with compound **III**, in solvent from methanol, 2-methoxyethanol, DMSO and DMF. The said reaction gave highest conversion (45.10% HPLC purity) in 2-Methoxyethanol as solvent with 2.55eq Ca DMPOEA HCl at 50-55°C. The concern for low purity of the crude compound **V**, is due to competitive reaction resulting in many side reactions. The said concern was overcome by making calcium free DMPOEA, which increases the solubility there by giving ~80% purity of the crude compound **V** in 2-methoxy ethanol as most suitable solvent.

To further purify compound **V**, different resins with differently modified counter ions were evaluated. Initially we attempted purification using strong anion exchange resin with formate as counter ions. The fractions did not contains product but shows number



Scheme 3 — Preparation of Desmethyl Citicoline



Scheme 4 — Preparation of Desmethyl Citicoline

of unknown peaks. Compound **V**, was further purified using weakly anion exchange resin using chloride, hydroxide, formate and acetate as counter ions. Based on the screening weakly anion exchange resin with formate as counter ion gives good separation. The Fractions with 95% plus purity were concentrated and converted to its sodium salt.

The said compound shows additional signal due to use of 2-methoxy ethanol and formic acid in proton NMR, might be due to use of formic acid during sodium salt formation.

The said residual impurities were eliminated by filtering reaction mass after completion and washing with sufficient amount of acetone to get rid from high boiling 2-methoxy ethanol. The crude product was purified by anion exchange resin with formate as counter ion and fractions were charcoalized to remove ester (**IV**) and cytidine-5′-monophosphate (**II**). Obtained product is submitted for NMR, confirms identity of the product.

Additionally a linear synthetic methodology was approached there by removing ion-exchange chromatography as disclosed in Scheme 4.

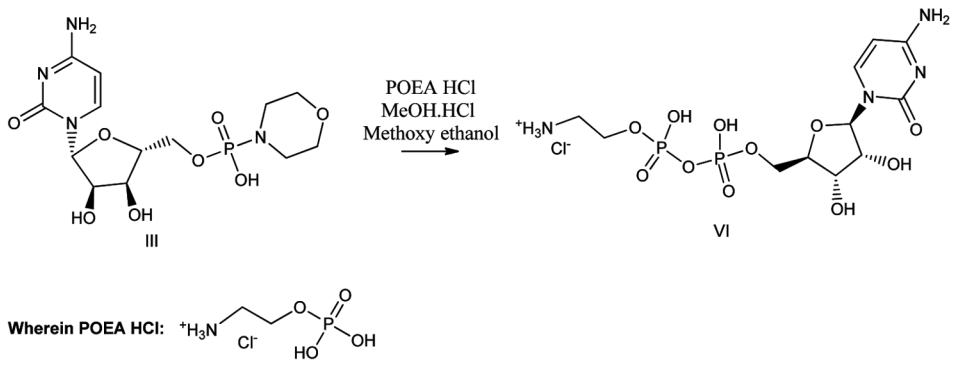
The process involves preparation of N-benzyl-N, N-dimethyl ethanol amine hydrochloride (**6**) as per the reported procedure by Wurm *et al*.[21] Which was treated with phosphorous oxychloride in *o*-dichlorobenzene and isolated as calcium salt of N-Benzyl-N, N-dimethyl amino ethyl phosphonic acid (**7**). Phosphonic acid derivative was reacted with Cytidinie-5′-morpholidate to give N-Benzyl desmethyl citicoline calcium (**8**). On catalytic debenzylation yields desmethyl citicoline calcium (**9**). Further purification, by treatment with monoisopropyl amine oxalate gives desmethyl citicoline (**V**). Obtained product was purified from methanol-acetone to afford white solid of product with purity by liquid chromatography 98% area normalization.

Cytidine 5′-diphosphoethanolamine, VI

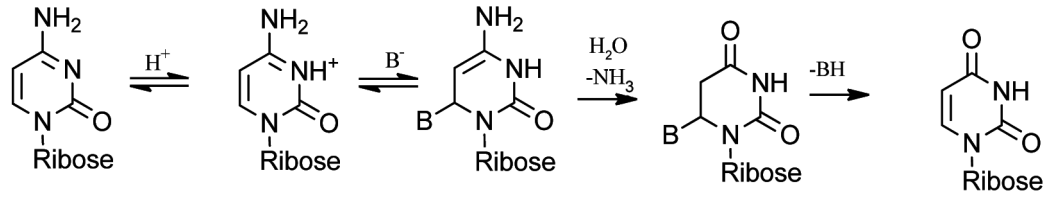
Cytidine-5′-diphosphoethanolamine is a listed impurity for Citicoline (USP), the synthesis we attempted again in line of Desmethyl Citicoline.

The calcium salt of 2-(phosphonooxy) ethanaminium chloride (Ca POEA. HCl) was prepared from amino ethanol instead of N, N-dimethyl amino ethanol (Scheme 5). Both Ca POEA. HCl and POEA HCl were treated with cytidine-5′-phosphomorpholidate in solvent from methanol, 2-methoxy ethanol and DMF to optimize the reaction conditions with screening for quantity of Ca POEA. HCl and POEA HCl. The experimental results reveal 2-methoxy ethanol as solvent and 8 equivalent of POEA HCl at 50-55°C gave 50% purity and was remained the best conversion and yield concern.

To improve the purity at crude stage for the said product, we reinvestigated the subject with all possible methodologies as making activation of cytidine-5′-monophospate using tosyl chloride[3], Cytidine-5′-phosphoropiperidates[19], Cytidine-5′-phosphoromorpholidates[5]. With our hands we were not able to get conversion more than 40% for the said impurity. With all the methodologies, very low conversion is due to the unreacted cytidine-5′-monophosphate. Due to low conversion, we proceed further with purification of the crude obtained from experimental disclosed using POEA HCl in 2-methoxy ethanol as disclosed above.



Scheme 5 — Preparation of Cytidine 5′-diphosphoethanolamine



Scheme 6 — Pathway for formation for Uridine-5′-diphosphocholine from Cytidine-5′-diphosphocholine

The crude compound was purified using weakly basic anionic resin (formate form) gave the product with ~91% purity with major impurity of cytidine-5′-morphplidate (~4%). Which was further purified by treating with diisoproylamine oxalate in water to get ~96% purity with eliminating the above said morpholidate impurity.

Uridine 5′-diphosphocholine, VII

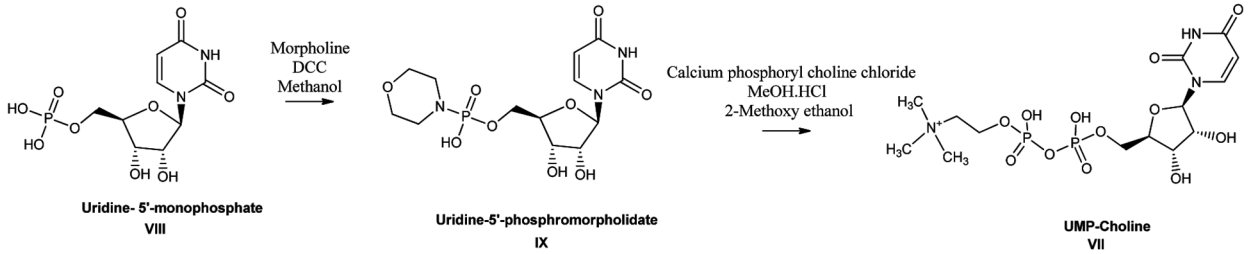
The said impurity may be formed due to deamination of Citicoline in-line of reported by Shapiro and klein for cytocine to uracil[24]. The hydrolytic deamination of cytidine to uridine is a slow reaction, with 16 hrs being required for half-reaction in 2.0 M citrate buffer at *p*H 4.0 and 95°C. The reported pathway for deamination is illustrated as Scheme 6.

Another approach for deamination cytidine to uridine is reported by using bisulfite solution[25], but said approach is not applicable due to the acidic and basic nature of the solution respectively and reflux conditions. The reaction when carried out using Citicoline, in both the conditions gave rise to cytidine-5′-monophosphate and Uridine-5′-monophosphate, confirming hydrolytic cleavage of diphosphate linkage.

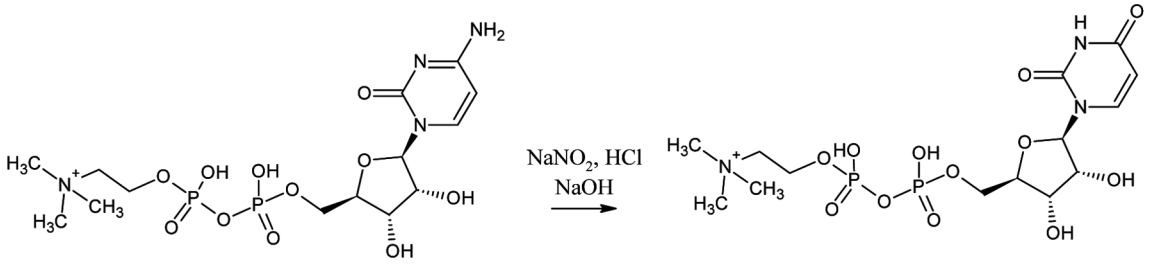
The synthesis of said compound is also disclosed by Sun *et al.*[19] Process involves condensation of uridine 5-phosphoropiperidates with choline chloride. Major concern was with respect to preparation of uridine 5-phosphoropiperidates, involves reaction of uridine-5′-monophosphate with piperidine in presence of 2, 2’-dithiodianiline[23]. Use of thiophenol and piperidine greatly limits its usage as are foul smelling. Herewith approach was to carry out reaction with an intention to provide direct and ease of handling chemistry for the synthesis of Uridine-5′-diphosphocholine.

Further experimentations were planned based by activation of uridine monophosphate using *para*-toluene sulfonyl chloride as reported by Kikugawa *et al.*[26] The reaction in-process shows conversion but during isolation we were not able to get clean conversion, with major being uridine-5′-monophosphate (**VIII**). We investigated the synthesis with an extension of procedure for Citicoline from cytidine-5′-monophsophate disclosed by Timmanagouda[8], wherein uridine-5′-monophosphate (**VIII**) was used as starting material and reaction scheme is disclosed in Scheme 7.

The process involves reaction of uridine-5′-monophosphate (**VII**) with morpholine in presence of DCC to yield Uridine-5′-phosphomorpholidate (**IX**). Which was concentrated to remove methanol and was taken in 2-methoxy ethanol for further condensation with Calcium phosphoryl choline chloride resulting desired compound. The process was optimized with



Scheme 7 — Preparation of Uridine 5′-diphosphocholine



Scheme 8 — Preparation of Uridine 5′-diphosphocholine from citicoline

respect to equivalent of calcium choline chloride and solvent of choice. Maximum conversion obtained with 3.25 equivalent of DCC and 2 equivalent of morpholine for Uridine-5′-phoshomorpholidate and subsequently giving 72% conversion by HPLC with unreacted uridine-5′-morpholidate (18%). The said crude compound was purified by ion exchange chromatography using weakly basic anion exchange resin (acetate form) to yield product as white solid with 96% HPLC purity.

Further to this methodology was extended from prior literature by Kikugawa *et al*.[26] and Daohuai and Tonghe[27] for preparation of uridine 5′-monophosphate from Cytidine 5′-monophosphate. The methodology was applied for Citicoline and to our surprise, even though this acidic nature of reaction media gave desired product **VII**, with purity 96% with only major impurity being Uridine 5′-monophosphate impurity **VIII** (8% area normalization by HPLC) (Scheme 8).

The crude compound was purified on weakly basic ion exchange resin (formate form) to give pure compound and was further converted to its sodium salt using aqueous sodium hydroxide and precipitation with acetone.

Conclusion

In summary, herewith we report an efficient and simple synthesis methodologies and purification process for pharmacopeia enlisted impurities of Citicoline and Citicoline sodium. All the impurities even though are structurally in close proximity of Citicoline, we are able to isolate disclosed impurities and are characterize to confirm their structure. The successful strategy involves DCC based activation of Cytidine-5′-monophosphate/ Uridine-5′-monophosphate. Further to this we herewith also developed a parallel methodologies for uridine and desmethyl impurities without use of any chromatographic purification.

Acknowledgement

The authors wish to thank Mr. Devesh Malladi, Mr. Aadya Malladi and the management of Embio Limited for their willing support and constant encouragement.

Supplementary Information

Supplementary information is available in the website http://nopr.niscpr.res.in/handle/123456789/58776.

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