Deprotection of N1-Methyladenosine-Containing RNA Using Triethylamine Hydrogen Fluoride

A. Apostle[1] and S. Fang[1]

[1]Department of Chemistry, and Health Research Institute,
Michigan Technological University,
Houghton, Michigan

Abstract

The N^1-methyladenosine (m^1A) epigenetic modification exists in many RNAs and is related to many human diseases. Chemically synthesized RNAs containing the modification are required for projects aimed at studying biological processes, mechanisms, and pathogenesis related to m^1A. Existing methods for the synthesis of m^1A containing RNAs use tetrabutylammonium fluoride (TBAF) for the deprotection of the 2'-silyl protecting groups. Since TBAF is non-volatile, and is relatively non-polar, its use in the desilylation of RNA requires repeated desalting, which is tedious and gives low yields. Here we report the use of the volatile and neat triethylamine hydrogen fluoride (TEA-HF) for desilylation of m^1A RNA synthesis. We found that the method is much more simple, and – in our hands - give significantly higher yield of RNA. Two major concerns for m^1A RNA synthesis are depurination and Dimroth rearrangement. HPLC and MALDI MS of the RNA indicated that depurination is not a problem for the new method. The absence of Dimroth rearrangement is proven by RNA digestion followed by HPLC analysis of the nucleosides.

Key Words: Deprotection, Dimroth rearrangement, epigenetics, N1-methyladenosine, RNA synthesis

1. Introduction

Over a hundred RNA modifications have been discovered in nature.[1] Among them, the N^1-methyladenosine (m^1A) modification and several others disrupt canonical base pairing and thus are particularly interesting. Given the biological significance of m^1A containing RNAs,[2] simple, reliable, and high yielding procedures for incorporating m^1A into RNAs could benefit many biomedical research projects. Synthesis of m^1A containing RNA has been reported, but the method for deprotection and purification is tedious and – in our hands - gives low yield. The synthesis uses standard phosphoramidite chemistry, and the m^1A modification is introduced using the chloroacetyl protected monomer.[3] The amino groups of the other monomers are protected with acyl groups, the phosphate is protected with a 2-cyanoethyl group, and 2'-OH is protected with silyl groups (TBDMS or TOM). If RP HPLC is used for RNA purification, the 5'-DMTr group is retained. Deprotection is achieved in two steps. First, the acyl and 2-cyanoethyl groups are removed under basic, non-aqueous, conditions. This also cleaves the RNA from the solid support. To prevent Dimroth rearrangement (Figure 1. A.),[4,5] which converts m^1A to N^6-methyladenosine (m^6A), typical conditions involving ammonium hydroxide at elevated temperature are avoided. Instead, the milder conditions consisting of 2 M ammonia in anhydrous MeOH at room temperature for 60 hours are used.[6] Second, the silyl groups are removed with TBAF. To prevent potential depurination (Figure 1. B.), the more acidic TEA-HF desilylation conditions[7] are avoided, even though RNA depurination is 100-1000 times slower than DNA.[8] This avoidance may be based on the consideration of the increased depurination rate caused by the alkylation of N-1 in m^1A. After all protecting groups are removed, the RNA is desalted and purified with reversed phase chromatography. Removal of

the 5'-DMTr group by an acid gave the RNA product. The tediousness and low yield of the procedure is caused by the use of TBAF.[6,9] Tetrabutylammonium (TBA) carries a permanent charge, and must be removed from RNA by repeated desalting procedures. In this paper, we report the synthesis of m^1A containing RNA and the use of TEA-HF instead of TBAF for desilylation. We found that the new method is more convenient, does not require repeated desalting, and gives higher yield of RNAs.

2. Materials and Methods

2.1. General procedures

RP-HPLC was performed on a JASCO LC-2000 Plus System: Pump, PU-2089 Plus Quaternary Gradient; detector, UV-2075 Plus; column for RNA purification, C-18 analytical (5 μm diameter, 100 Å, 250 × 4.6 mm), and for digestion analysis, CORTECS C18 2.7μm, 2.1x50mm. Gradient for RNA purification[10]: Buffer A, 0.1 M triethylammonium acetate, 5% acetonitrile; Buffer B, 90% acetonitrile; Buffer B (0-45%) in Buffer A over 60 min at 1 mL/min. Gradient for analysis of nucleosides from enzymatic digestion of RNAs **1a** and **1b**[11]: Buffer A, 5 mM NH_4OAc, pH 6.0; Buffer B, 40% ACN (v/v); Buffer B (0-60%) in Buffer A over 100 min at 0.85 mL/min. All profiles were generated by detecting absorbance at 260 nm. MALDI MS was obtained on a Bruker MALDI-TOF Microflex LRF mass spectrometer using negative mode, 0.139 laser beam attenuation, 30 Hz laser repetition rate, 100 shots for each spectrum, 100 ns delay time, and 19 kV and 15.55 kV ion source voltages. ORN samples were prepared on parafilm by mixing desalted ORN solution (0.5 μL) with a matrix solution (0.5 μL), which was saturated 3-hydroxypicolinic acid (3-HPA) in the solvent mixture containing equal volumes of ACN and 0.1% aqueous TFA with 10 mg/mL diammonium hydrogen citrate. The solution was loaded onto a stainless steel MALDI substrate, then allowed to air dry and crystallize.

2.2. RNA synthesis

RNAs (30-mer **1a**: 5'-CUC GUm^1A GAC UAU AAC UUA AUC UCA CAU AGC. 30-mer **1b**: 5'-CUC GUm^6A GAC UAU AAC UUA AUC UCA CAU AGC) were synthesized on a MerMade 6 DNA/RNA synthesizer at 1 μmol scale using standard phosphoramidite chemistry. A universal solid support (BioAutomation Cat# MM1-3500-1) was used. Deblocking: TCA (2% in DCM), 90 sec × 3. Coupling: 5'-DMTr-2'-TBDMS-CE-phosphoramidites of Bz-A, Ac-C, Ac-G, U, Cl-Ac-m^1A (for RNA **1a**) and Pac-m^6A (for RNA **1b**) (0.1 M in ACN), 5-(ethylthio)-1*H*-tetrazole (ETT) (0.25 M in ACN), 6 min × 3 or 15 min × 3 (for m^1A only). Capping: Cap A THF/pyridine/Ac_2O, Cap B Methylimidazole (16% in THF), 50 sec × 3. Oxidation: I_2 (0.1 M in THF/pyridine/H_2O), 40 sec × 3. DMTr at the 5' end of RNA was kept.

2.3. ORN cleavage and deprotection

For RNA **1a**, the CPG was incubated in a solution of NH_3 in CH_3OH (2.0 M) at rt for 60 h. The supernatant was transferred to a sterile centrifuge tube, and the CPG was washed with 2.0 M NH_3 CH_3OH (washing with water was avoided to prevent potential Dimroth rearrangement caused by hydroxide ions). The supernatant and washes were combined, and volatiles were evaporated using a vacuum centrifugal evaporator. For the TEA-HF deprotection method, to the RNA was added DMSO (115 μL). The mixture was heated at 65 ºC for 5 min to assist dissolution. Triethylamine (TEA, 60 μL) was added. After mixing, TEA-3HF (75 μL) was added. The mixture was heated at 65 ºC for 2.5 h, then cooled on ice, whereupon GlenPak™ RNA Quenching Buffer (1.75 mL) was added and mixed well. The solution was then loaded onto a GlenPak™ RNA purification cartridge (for use with disposable syringes, Cat# 60-6200) that was preconditioned using ACN (0.5 mL) followed by TEAA (2 M, 1.0 mL). The cartridge was washed sequentially with the mixture of ACN and 2 M TEAA solution (1:9 v/v, pH 7.0, 1.0 mL), RNase free water

(1.0 mL), TFA (2%, 1.0 mL × 2, 5'-DMTr deprotection), and RNase free water (1.0 mL × 2). The fully deprotected RNA was then eluted with the solution of NH_4HCO_3 (0.1 M in 30% ACN, 1.0 mL). The solution was evaporated to dryness. For the TBAF deprotection method, the residue from NH_3-treated RNA was incubated in TBAF (1 M in THF, 1.5 mL) at rt for 18 h. The reaction was quenched with NH_4OAc (1.5 M, 1.0 mL). The mixture was desalted twice using Glen GelPak™ 2.5 Desalting Column with water as the eluent. The fractions containing RNA (first 2.5 mL eluted) were combined and evaporated using a vacuum centrifugal evaporator. The residue was purified using RP HPLC to separate the full length DMTr-ON RNA from the DMTr-OFF failure sequences. The purified full length DMTr-ON RNA was then treated with 80% AcOH to remove the DMTr group, and volatiles were removed under vacuum. For RNA **1b**, standard conditions involving 30% NH_4OH and 40% CH_3NH_2 (1:1 v/v) at 65 °C for 20 min (for cleavage and deprotecting acyl and 2-CE groups) and TEA-3HF at 65 °C for 2.5 h (for desilylation) were used. The RNA was purified with GlenPak™ cartridge. All the RNAs (**1a**-**b**) were further purified with RP HPLC, and analyzed for purity with RP HPLC, and MALDI MS.

2.4. Enzymatic digestion of RNAs and dephosphorylation of m^6ATP

A solution of RNA **1a** (500 ng), 1× Nuclease P1 Buffer (NEB Cat# B0660) and Nuclease P1 (60 U, NEB Cat# M0660S), in a final volume of 6 µL, was incubated at 37 °C for 1 h. The nuclease was inactivated by heating to 75 °C for 10 min. After allowing the solution cool to r.t., 1× FastAP™ Thermosensitive Alkaline Phosphatase buffer (ThermoFisher, Cat# EF0654) and FastAP™ Thermosensitive Alkaline Phosphatase (0.6 U, ThermoFisher, Cat# EF0654) was added (7.5 µL final volume). The solution was incubated at 37 °C for 2 h. The phosphatase was inactivated by heating at 75 °C for 10 min. The nucleosides were analyzed with RP HPLC. RNA **1b** was digested and analyzed similarly.

To a solution containing 100 nmole of m^6ATP (TriLink Biotech., SKU N-1013-1), there was added 1× FastAP™ Thermosensitive Alkaline Phosphatase buffer, and FastAP™ Thermosensitive Alkaline Phosphatase (1 U), achieving a final volume of 10 µL. The phosphatase was inactivated by heating to 75 °C for 10 min.

3. Results and Discussion

The 30-mer RNA **1a** containing the m^1A modification was synthesized on CPG using standard phosphoramidite chemistry. The m^1A nucleotide was incorporated using the chloroacetyl protected phosphoramidite. The 5'-DMTr group was kept. To prevent Dimroth rearrangement, which converts m^1A to m^6A in the presence of hydroxide, cleavage of the RNA from CPG and removal of *exo*-amino acyl and 2-cyanoehtyl (2-CE) protecting groups were achieved using reported conditions involving 2.0 M NH_3 in CH_3OH at rt for 60 h. To compare our proposed desilylation method with TEA-3HF with the reported TBAF method,[9] CPG of the fully synthesized and DMTr tagged RNA was partitioned into five equal portions. Each was deprotected and cleaved from solid support using the same conditions referenced above. One portion was treated with TEA-3HF. The mixture was loaded onto a GlenPak™ RNA purification cartridge. Impurities including failure sequences were removed by washing. The more hydrophobic full-length RNA, which has a 5'-DMTr group, stayed on the cartridge. After cleaving the DMTr group with 2% TFA, the full-length RNA was eluted with the volatile buffer NH_4HCO_3 in 30% ACN. The RNA was further purified with RP HPLC. MALDI-TOF MS analysis gave correct molecular weight, with no detection of depurination. HPLC and MS analyses are provided in the supporting information. Another potion of the RNA was desilylated using TBAF following the aforementioned procedure (this specific method being chosen due to it being a standard commercial approach and universally accessible method).[9] The excess TBAF and other small molecule impurities was removed using Glen GelPak™ 2.5 Desalting Column, the

separation principle of which is size-exclusion chromatography. Due to the large amount of non-volatile salts, the desalting had to be carried out two or more times, which was the cause of the tediousness and low RNA yields of the TBAF method. For a comparison of the RNA yields of the two desilylation methods, the method using TEA-3HF gave 2.4 μg RNA per milligram of CPG while the method using TBAF gave 0.03 μg RNA per milligram of CPG. The desilylation procedures for the TEA-3HF method and the TBAF method were repeated for an additional one and two times, respectively. The large difference of yields between the two methods was consistent.

Because m^1A and - its Dimroth rearrangement product - m^6A, have the same molecular weight, MALDI MS cannot determine if rearrangement of m^1A in RNA **1a** had occurred during the TEA-3HF desilylation procedures. For this reason, we synthesized RNA **1b**, which has the same sequence as **1a** with the exception that m^1A is replaced by m^6A (Figure 2). The two RNAs were digested by nuclease and dephosphorylated by phosphatase. The resulting nucleosides were analyzed with RP HPLC. As shown in Figure 3, the m^6A nucleoside has a retention time about 11 minutes (Figure 3. A.), while that of m^1A has a retention time of about 1 minute, owing to its likely cationic form (Figure 3. B.). To further prove the retention time of m^6A, the HPLC profile of an m^6A nucleoside (obtained by the dephosphorylation of an m^6ATP standard, as explain in the methods section above) was generated (Figure 3. C.). It is clear that the digestion of RNA **1a** does not contain any m^6A. Therefore, we conclude that m^1A in **1a** was stable under the newly applied TEA-3HF desilylation conditions, and RNA **1a** was pure.

4. Conclusion

In order to develop a more convenient and higher yielding method for the production of m^1A containing RNAs, the use of TEA-HF as a silyl deprotecting agent for RNA deprotection was studied. We found that the new conditions did not cause m^1A depurination nor Dimroth rearrangement, and pure m^1A containing RNA can be readily obtained. Compared with existing desilylation conditions using TBAF, the newly applied method is far more convenient and reliable, and in our hands gives much higher RNA yield. We expect that the new method will find use for projects that require chemically synthesized m^1A containing RNAs in the research field of epigenetics, and beyond.

Funding

Financial support from NSF (1954041), NIH (GM109288), Robert and Kathleen Lane Endowed Fellowship (A.A.), and HRI Fellowship (A.A.); and NSF equipment grants (2117318, NMR; 1048655 & 1531454, MS); are gratefully acknowledged.

Disclosure Statement

The authors hereby declare no competing interests.

References

1) Netzband, R.; Pager, C.T. Epitranscriptomic marks: Emerging modulators of RNA virus gene expression. *WIREs RNA*. **2020**, 11, e1576. DOI: 10.1002/wrna.1576.
2) Jonkhout, N.; Tran, J.; Smith, M.A.; Schonrock, N.; Mattick, J.S.; Novoa, E.M. The RNA modification landscape in human disease. *RNA*. **2017**, 23, 1754-1769. DOI: 10.1261/rna.063503.117.

3) Efimtseva, E.V.; Mikhailov, S.N.; Rozenski, J.; Busson, R.; Van Aerschot, A.; Herdewijn, P. Chemical Incorporation of 1-Methyladenosine, Minor tRNA Component, into Oligonucleotides. *Nuc., Nuc. & Nuc. Acids*. **2003**. 22, 1113-1115. DOI: 10.1081/NCN-120022749.
4) Dimroth, O. Ueber intramolekulare Umlagerungen. Umlagerungen in der Reihe des 1, 2, 3-Triazols. *Justus Liebigs Annalen der Chemie*. **1909**, 364, 183-226. DOI: 10.1002/jlac.19093640204.
5) Engal, J. D. Mechanism of the dimroth rearrangement in adenosine. *Biochem. Biophys. Res. Commun.* **1975**, 64, 581-586. DOI: 10.1016/0006-291X(75)90361-7
6) Mikhailov, S. N.; Rozenski, J.; Efimtseva, E. V.; Busson, R.; Van Aerschot, A.; Herdewijn, P. Chemical incorporation of 1-methyladenosine into oligonucleotides. *Nucleic Acids Researc.* **2002**, 30, 1124-1131. DOI: 10.1093/nar/30.5.1124.
7) Westmanu, E.; Stromberg, R. Removal of t-butyldimethylsilyl protection in RNA-synthesis. Triethylamine trihydrofluoride (TEA, 3HF) is a more reliable alternative to tetrabutylammonium fluoride (TBAF). *Nucleic Acids Res.* **1994**, 22, 2430-2431. DOI: 10.1093/nar/22.12.2430.
8) Jobst, K.A.; Klenov, A.; Neller, K.C.M.; Hudak, K.A. Effect of Depurination on Cellular and Viral RNA. In *Modified Nucleic Acids in Biology and Medicine*. Springer, Cham., 2016; 273-297. DOI: 10.1007/978-3-319-34175-0_12.
9) Glenn Research. Modified RNA Phosphoramidites Useful in siRNA Research and Biologically Significant 1-Methyl-Adenosine: 1-Methyl-Adenine in Nucleic Acids; The Glen Report: Sterling, Virginia, 2007; volume 19, number 1, 1-5. https://www.glenresearch.com/reports/gr19-1.
10) Pokharel, D.; Fang, S. Polymerizable phosphoramidites with an acid-cleavable linker for eco-friendly synthetic oligodeoxynucleotide purification. *Green Chem.* **2016**, 18**,** 1125-1136. DOI: 10.1039/C5GC01762A.
11) Nees, G.; Kaufmann, A.; Bauer, S. Detection of RNA modifications by HPLC analysis and competitive ELISA. *Methods Mol Biol*. **2014**, 1169, 3-14. DOI: 10.1007/978-1-4939-0882-0_1.

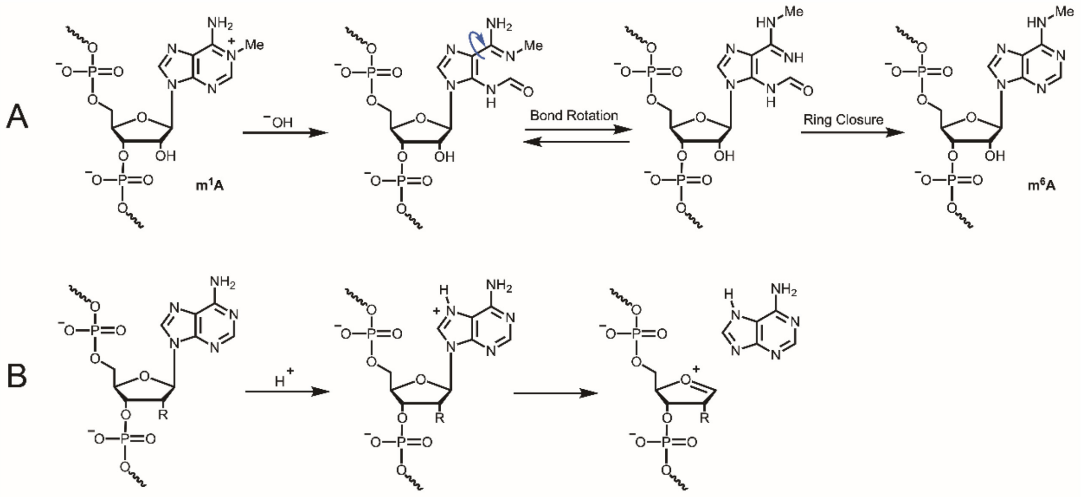

Figure 1. Potential degradation of adenosine, and its derivatives, in RNA: (**A**) Dimroth rearrangement, and (**B**) depurination.

RNA 1a: 5' - CUC GUm^{1}A GAC UAU AAC UUA AUC UCA CAU AGC - 3'

RNA 1b: 5' - CUC GUm^{6}A GAC UAU AAC UUA AUC UCA CAU AGC - 3'

Figure 2. RNA oligos synthesized.

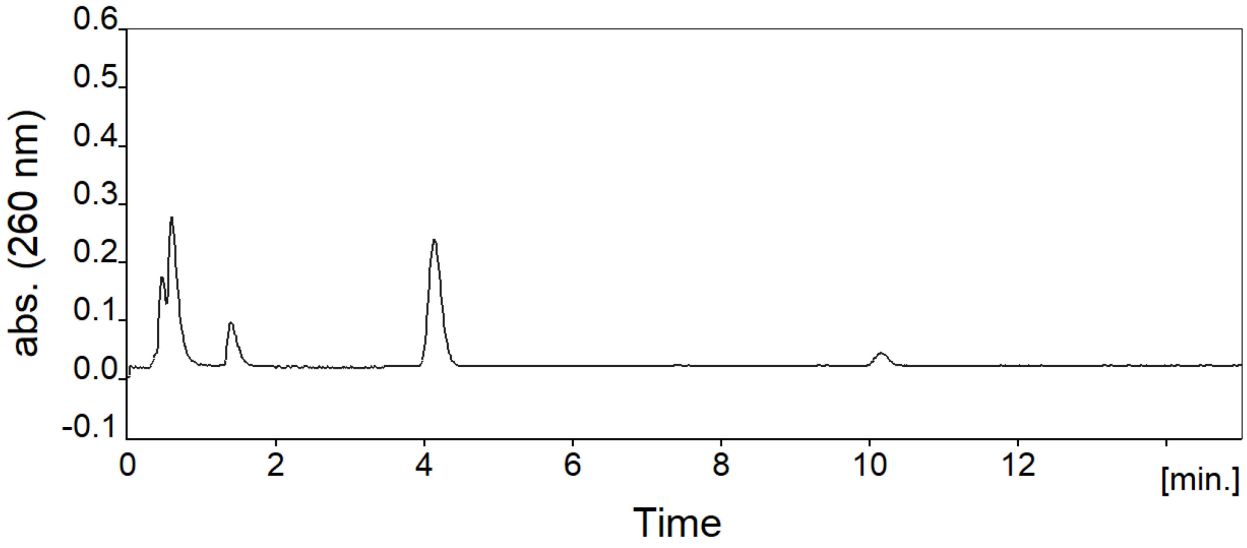

Figure 3. A. RP HPLC analysis of 200 ng RNA 1b nuclease and dephosphorylase digestion.

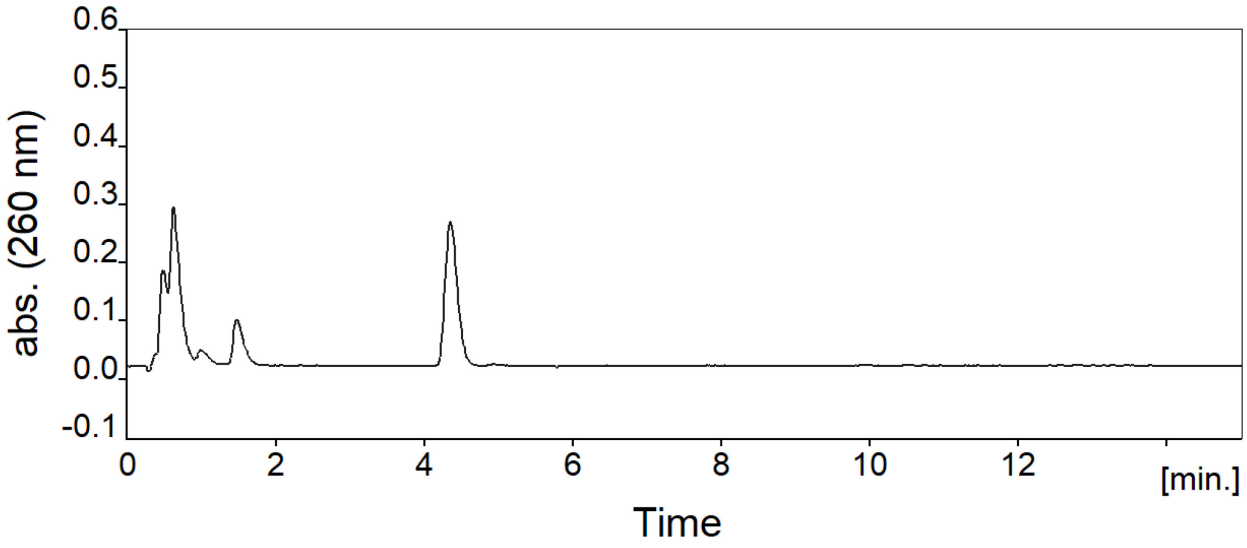

Figure 3. B. RP HPLC analysis of 200 ng RNA 1a nuclease and dephosphorylase digestion.

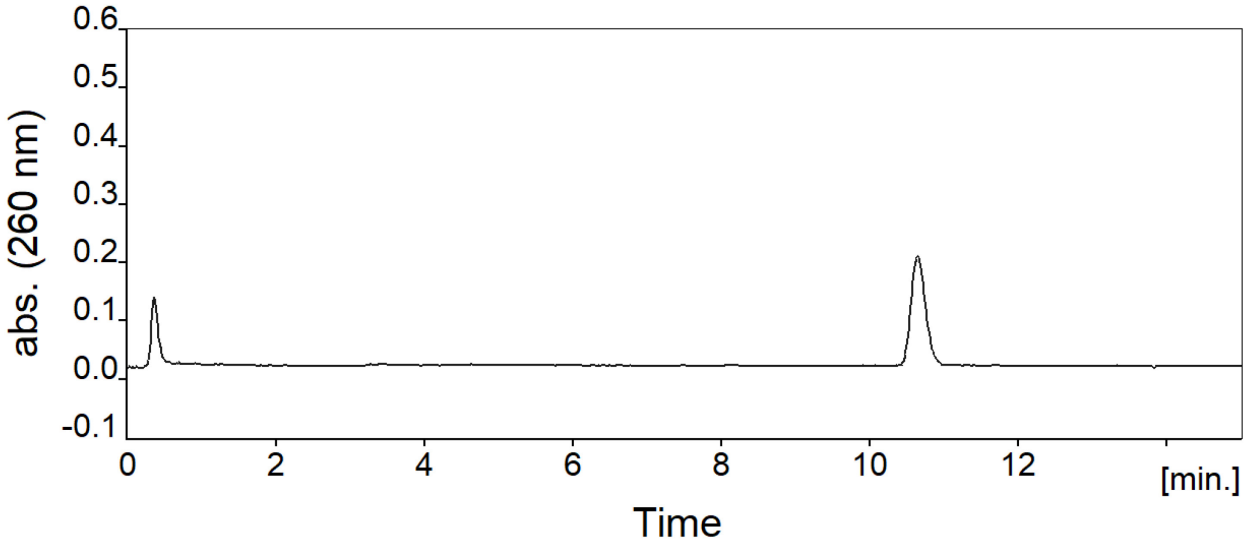

Figure 3. C. RP HPLC analysis of 0.2 nmole m^6A.