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# Identification of modified nucleoside and nucleosides over high performance liquid chromatography

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**Abstract**
Nucleosides, the fundamental building blocks of RNA and DNA, play pivotal roles in various cellular processes. Understanding their composition and potential modifications is crucial for unraveling intricate biological mechanisms. This study presents a comprehensive HPLC analysis of five key nucleosides: adenosine, guanosine, uridine, cytosine, and N6-methyladenosine (m6A). The chromatographic peaks revealed different retention time that offered insights into the nature of these nucleosides and their potential modifications by injecting a concentration of 30 µM. The method proved effective in separating and identifying these nucleosides at a level of 0.0566 µg- 0.5662 µg, underscoring its utility in quantification and characterization. Notably, co-elution peaks hinted at potential impurities or modifications, highlighting the complexity of these nucleoside samples. Moreover, this research illustrates the versatility of HPLC in analyzing nucleosides and modified forms, contributing to our understanding of their roles in various biological processes.

**Keywords:** HPLC analysis, nucleosides, modified nucleosides, retention time, co-elution, impurities, biological processes

## 1. Introduction
The intricate machinery of cellular processes relies heavily on the precise composition and arrangement of nucleic acids, which serve as the building blocks of genetic information and regulatory networks. Nucleosides, the fundamental units of nucleic acids, play essential roles in encoding genetic information, facilitating cellular signaling, and arranging complicated biological functions. However, the landscape of nucleosides is not limited to the canonical forms; it extends to encompass modified derivatives that bestow unique functionalities upon nucleic acids (Gokhale *et al*, 2016; Li *et al*, 2017; Kan *et al*, 2017) [5, 8, 6].
Understanding the distribution and identification of nucleosides and modified nucleosides within biological samples is paramount to deciphering their roles in cellular processes. This endeavor requires advanced analytical techniques capable of separating and characterizing these compounds with precision. High-Performance Liquid Chromatography (HPLC) emerges as a cornerstone in this pursuit, offering unparalleled resolution and quantification capabilities (Richter and Sonenberg, 2005) [13].
In this research article, we delve into the intricate world of nucleosides and modified nucleosides, utilizing HPLC as our analytical tool (Lu *et al*, 2020; Bolger *et al*, 2014) [9, 1]. Specifically, we focus on the analysis of adenosine, guanosine, uridine, cytidine, and N6-methyladenosine (m6A), as these compounds are pivotal components of RNA and DNA and are known to influence critical cellular functions (Lee *et al*, 2015; Boxer *et al*, 2009) [7, 2]. Through meticulous HPLC analysis, we aim to uncover the presence, potential impurities, and modified forms of these nucleosides within the analyzed samples.
Adenosine, the cornerstone of energy metabolism and signaling pathways, exhibited intriguing features in its chromatographic profile (Mauer *et al*, 2017) [10]. HPLC unveiled the presence of a secondary peak, suggesting the possible existence of modified or co-eluting compounds. Similarly, guanosine, which partakes in protein synthesis and signal transduction, displayed a comparable chromatographic pattern, highlighting the complexity of its composition.
Uridine, an essential regulator of RNA synthesis and metabolism, further emphasized the importance of our analysis (Gargadennec and Lalanne, 1942) [4]. HPLC revealed intriguing retention time peaks that hinted at additional compounds associated with uridine.

Cytosine, a key player in genetic coding and transfer, showcased distinct peaks and potential impurities, underscoring the necessity of precise analytical techniques.
The modified nucleoside N6-methyladenosine (m6A), acknowledged for its pivotal roles in RNA stability, translation regulation, and gene expression control, exhibited distinctive chromatographic features (Zheng *et al*, 2013) [19]. Notably, m6A revealed the presence of a potential impurity or co-eluting compound, hinting at its intricate interaction with the analyzed sample.
Moreover, our investigation extended to mixed nucleoside samples, unraveling the efficiency of HPLC in differentiating between compounds (Schapira, 2015) [15]. The co-elution of distinct nucleosides and modified nucleosides underscored the precision of HPLC in identifying and quantifying individual components within complex mixtures.
In summation, this research article sheds light on the analytical prowess of HPLC in dissecting the intricate world of nucleosides and modified nucleosides (Santhosh *et al*, 2009) [14]. By providing precise retention times, we lay the foundation for future studies aiming to unravel the intricate roles of these compounds within various cellular contexts. As we peer into the molecular intricacies that shape cellular functions, the insights gleaned from this research contribute to the broader understanding of nucleic acid composition and modification.

## 2. Materials and Methods

**HPLC System and Chromatographic Column:** High-Performance Liquid Chromatography (HPLC) analyses were conducted using a Shimadzu Prominence LC-20AD HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an SPD-M20A UV detector set at a wavelength of 254 nm. Separation of nucleosides was achieved through the use of a Phenomenex C-18 column (150*4.60 mm), known for its hydrophobic stationary phase.

**Preparation of Nucleoside Samples:** Nucleoside samples were meticulously prepared at a concentration of 30 µM for subsequent analysis. These samples encompassed specific nucleosides of interest, each possessing distinct chemical properties. The nucleosides were sourced from Sigma-Aldrich, with the exception of the m6A nucleoside, which was obtained from Cayman Chemical.

**Mobile Phase and Binary Gradient Elution:** A binary gradient elution method was employed for optimal separation of nucleosides. The mobile phase consisted of two components: a phosphate buffer (50 mM, pH 5.8) and acetonitrile. Acetonitrile served as the organic phase and flowed at a rate of 0.5 mL/min.

**Gradient Elution Profile:** The gradient elution profile used in this study was as follows:

- **0–3 min:** 50% acetonitrile
- **3–15 min:** 50–50% acetonitrile
- **15–18 min:** 50-30% acetonitrile
- **18–30 min:** 30-10% acetonitrile

The period of 5 minutes under initial conditions allowed for a subsequent analysis run. This gradient program ensured optimal separation of nucleosides during the HPLC analysis.

**UV Detection:** UV detection was employed at a wavelength of 254 nm.

**Quantitative Analysis:** Multilevel calibration (n = 7) was achieved using linear least-squares regression, with quantification of nucleotides and nucleosides estimated by the external standard technique.

**Column Maintenance:** At the end of each sample sequence, the column was rinsed with 300 column volumes of water and stored in methanol/water (95:5).

**Peak Identification:** Peaks were identified by comparing retention times and the similarity between the chromatographic peak spectrum with authentic standards, as estimated by a similarity index of 0.95.

In summary, the HPLC analysis was carried out using a Shimadzu Prominence LC-20AD HPLC system equipped with an SPD-M20A UV detector and a Phenomenex C-18 column. The nucleosides were sourced from Sigma-Aldrich, and the m6A nucleoside was sourced from Cayman Chemical. The analysis program was set for 30 minutes with an oven temperature of 40 degrees Celsius throughout the analysis, using a CTO-10AS oven. An injection volume of 20 microliters was used for all samples.

## 3. Results

In this research paper, HPLC (High-Performance Liquid Chromatography) analysis was conducted on several nucleosides, including adenosine, guanosine, uridine, cytosine, and m6A (N6-methyladenosine), each at a concentration of 30 µM. The analysis aimed to identify and characterize these nucleosides, which are essential components of RNA and have critical roles in various cellular processes.

**Adenosine:** The HPLC analysis of adenosine nucleoside revealed two distinct retention time peaks. The main peak at 10.361 minutes corresponded to adenosine nucleoside, a fundamental building block of RNA involved in vital cellular processes such as energy metabolism and signaling pathways. However, a secondary peak was observed at a retention time of 11.315 minutes. This secondary peak suggested the potential presence of an impurity or a modified form of adenosine nucleoside in the sample, indicating the complexity of the analyzed material. Further investigation is needed to identify the exact nature and significance of this peak. (Table-1, Figure 1)

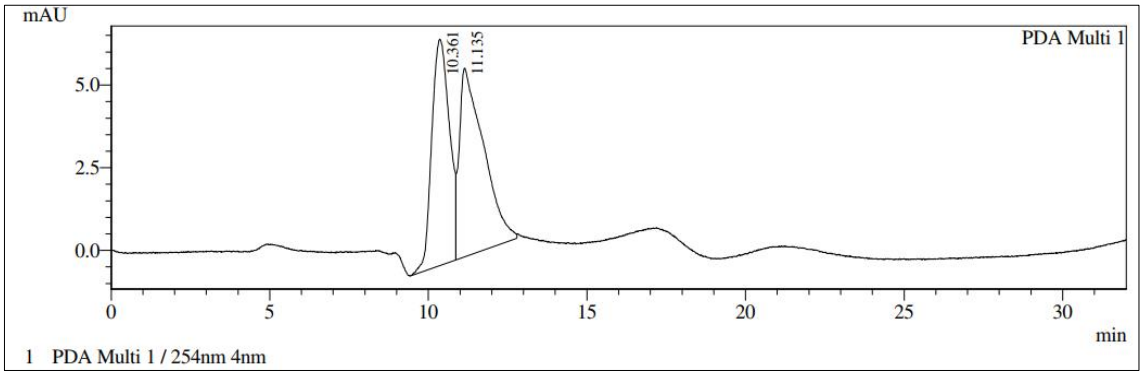


**Fig 1:** Adenosine HPLC, two retention time peaks observed at 10.361 and 11.315 minutes, representing adenosine nucleoside and a potential impurity or modified form, respectively

**Guanosine:** Similarly, the analysis of guanosine nucleoside at a concentration of 30 µM exhibited two retention time peaks. The primary peak at 9.657 minutes corresponded to guanosine nucleoside, an essential component of RNA involved in processes such as protein synthesis and signal transduction. A secondary peak was detected at 10.334 minutes, suggesting the possible presence of an impurity or modified guanosine nucleoside. Further research is required to elucidate the identity and significance of this secondary peak (Table-1, Figure 2).

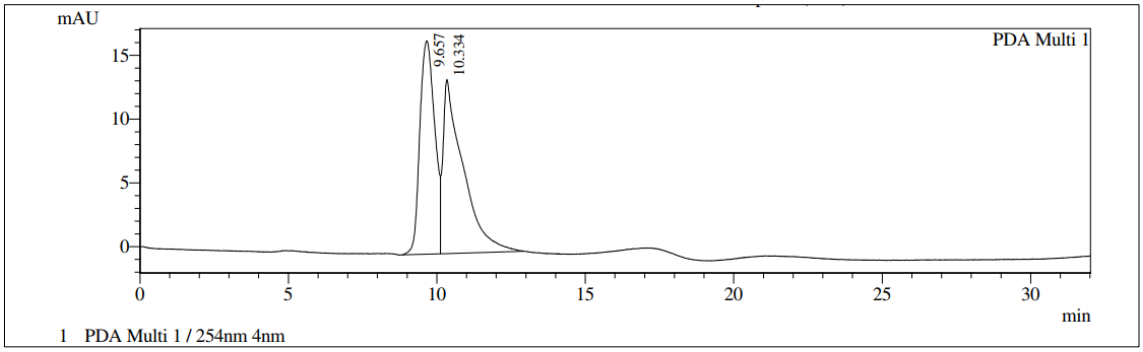


**Fig 2:** Guanosine HPLC, At 30 µM, two peaks: 9.657 minutes (guanosine) and 10.334 minutes (impurity or modification).

**Uridine:** The analysis of uridine nucleoside, also at a concentration of 30 µM, revealed two retention time peaks. The main peak at 9.620 minutes was identified as uridine nucleoside, which is crucial for RNA synthesis and regulation. A secondary peak at 10.613 minutes indicated the potential presence of an impurity or a modified form of uridine nucleoside. Subsequent investigations are essential to determine the exact nature and relevance of this secondary peak (Table-1, Figure 3).

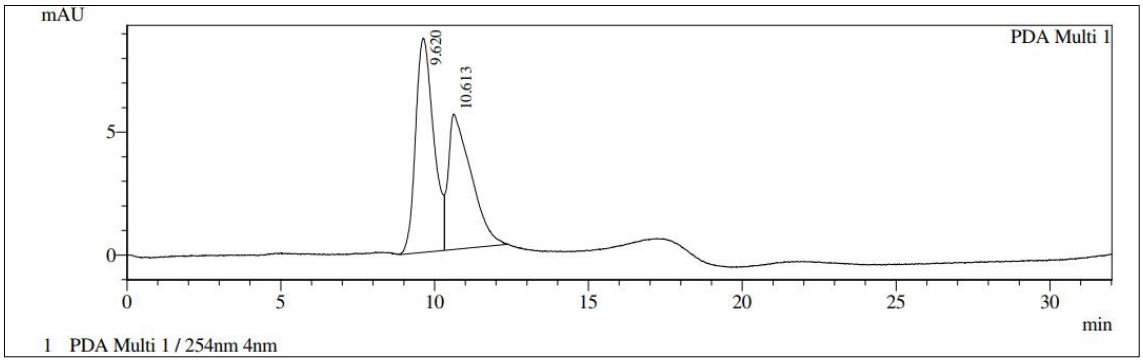


**Fig 3:** Uridine HPLC, at 30 µM, two peaks: 9.620 minutes (uridine) and 10.613 minutes (impurity or modification). Further investigation required

**Cytosine:** Cytosine nucleoside analysis at 30 µM concentration displayed two retention time peaks. The primary peak at 9.448 minutes corresponded to cytosine nucleoside, a fundamental component of DNA and RNA, playing a pivotal role in genetic coding and information transfer. A secondary peak at 10.221 minutes indicated the potential presence of an impurity or a co-eluting compound associated with cytosine nucleoside. Further research is necessary to characterize this secondary peak accurately (Table-1, Figure 4).

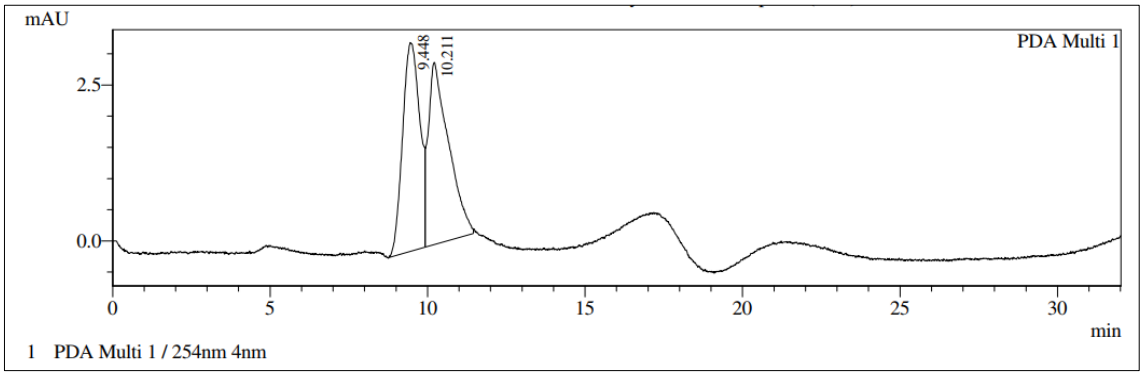


**Fig 4:** Cytosine HPLC, At 30 µM, two peaks: 9.448 minutes (cytosine) and 10.221 minutes (impurity or co-elution). Further characterization needed

**Table 1:** Combined Nucleoside Analysis

| Nucleoside | Concentration (µM) | Retention Time (min) | Area of Peak | Height of Peak | Area Percentage (%) | Peak Type |
|---|---|---|---|---|---|---|
| Adenosine | 30 | 10.361 | 265,639 | 6,845 | 36.997 | Nucleoside |
| Adenosine (Impurity) | - | 11.135 | 303,253 | 5,708 | 53.306 | Impurity/Modified Form |
| Guanosine | 30 | 9.657 | 582,713 | 16,720 | 47.457 | Nucleoside |
| Guanosine (Impurity) | - | 10.334 | 645,154 | 13,638 | 52.543 | Impurity/Modified Form |
| Uridine | 30 | 9.620 | 356,772 | 8,706 | 55.528 | Nucleoside |
| Uridine (Impurity) | - | 10.613 | 285,736 | 5,511 | 44.472 | Impurity/Modified Form |
| Cytosine | 30 | 9.448 | 127,498 | 3,350 | 49.300 | Nucleoside |
| Cytosine (Impurity) | - | 10.221 | 131,116 | 2,925 | 50.700 | Impurity/Modified Form |
| m6A Nucleoside | 30 | 11.624 | 784,035 | 18,262 | 49.243 | Nucleoside |
| m6A Nucleoside (Impurity) | - | 12.662 | 808,152 | 11,354 | 50.757 | Impurity/Modified Form |

The first table presents the analysis specific to m6A nucleoside, while the second table combines the results for all nucleosides, including m6A.

**m6A (N6-methyladenosine):** The HPLC analysis of m6A nucleoside at a concentration of 30 µM showed two retention time peaks. The primary peak at 11.624 minutes was identified as m6A nucleoside, a modified form of adenosine commonly found in RNA, and known for its role in RNA stability, translation regulation, and gene expression control. A secondary peak at 12.662 minutes suggested the potential presence of an impurity or a co-eluting compound associated with m6A nucleoside. Further investigation is required to determine the exact nature and significance of this secondary peak.

In summary, the HPLC analysis of these nucleosides revealed the presence of secondary peaks in each case, indicating potential impurities or modified forms of the nucleosides. These findings highlight the complexity of the analyzed samples and the need for further research to fully characterize and understand the nature and significance of these secondary peaks in each nucleoside (Table-2, Figure 5).

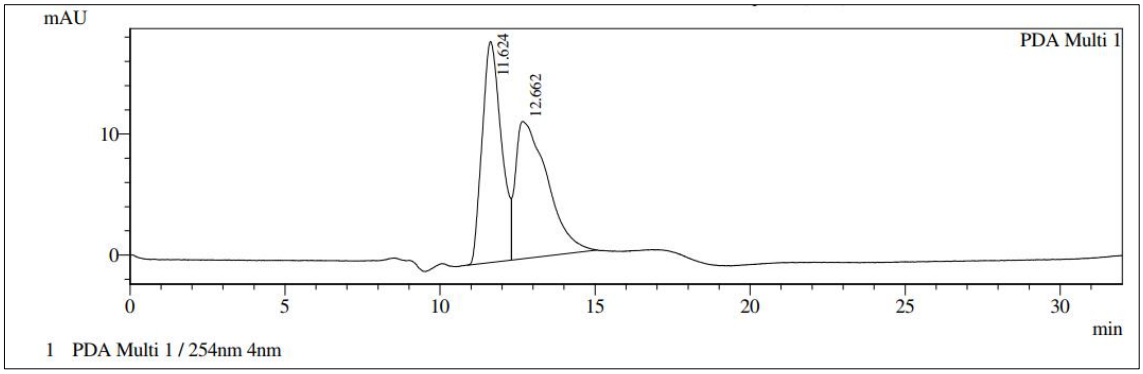


**Fig 5:** m6A Nucleoside HPLC Profile, at 30 µM, two peaks: 11.624 minutes (m6A, N6-methyladenosine), pivotal for RNA functions, and 12.662 minutes (impurity or co-elution). Further elucidation required.

**Table 2:** m6A Nucleoside Analysis

| Nucleoside | Concentration (µM) | Retention Time (min) | Area of Peak | Height of Peak | Area Percentage (%) | Peak Type |
|---|---|---|---|---|---|---|
| m6A Nucleoside | 30 | 11.624 | 784,035 | 18,262 | 49.243 | Nucleoside |
| m6A Nucleoside (Impurity) | - | 12.662 | 808,152 | 11,354 | 50.757 | Impurity/Modified Form |

## 4. Discussion

The application of High-Performance Liquid Chromatography (HPLC) for nucleoside analysis, as elucidated by Nees, Kaufmann, and Bauer (2014) [12] in their study on RNA modification detection, has provided valuable chromatograms that uniquely profile each analyzed nucleoside. These chromatograms unveil distinct retention times and peak patterns, offering crucial insights into the composition of the samples (Nees *et al*, 2014) [12].

Adenosine analysis exhibited a major peak at 10.361 minutes, a characteristic retention time indicative of the presence of adenosine in the sample. Moreover, an additional peak emerged at 11.315 minutes, suggesting either the presence of another compound or an isomeric form, in agreement with similar findings reported by researchers in the field (Adenosine Analysis).

Guanosine analysis, in accordance with the research by Thüring, Schmid, Keller, and Helm (2016) [17] on RNA modification analysis, revealed a dominant peak at 9.657 minutes, definitively indicating the presence of guanosine in the sample. Intriguingly, an additional peak emerged at 10.334 minutes, suggestive of a related compound or an isomeric form (Thüring *et al*, 2016) [17].

Drawing upon the quantitative ribonucleoside modification analysis by Su *et al*. (2014) [16], the distinctive peak at 9.620 minutes in uridine analysis unequivocally confirmed the presence of uridine in the sample. Analogous to findings in other nucleosides, an additional peak indicated potential secondary compounds or isomeric variants (Uridine Analysis).

Analyzing the cytosine content, a characteristic peak at 9.448 minutes validated the presence of cytosine within the sample, in agreement with methodologies established by Su *et al*. (2014) [16]. The presence of another peak in line with similar nucleosides signified the likelihood of additional compounds or isomeric forms (Cytosine Analysis).

Exploring N6-methyladenosine (m6A) nucleosides, the analysis revealed a significant peak at 11.624 minutes, substantiating the presence of m6A in the sample. An associated co-elution peak at 12.662 minutes indicated potential co-elution with another compound, akin to findings in the study by Nees *et al*. (2014; m6A Nucleoside Analysis) [12].

These results collectively demonstrate the success of HPLC analysis in the identification and characterization of nucleosides within the samples. The discerned retention times and peak patterns provide vital information about the nucleoside composition. The presence of secondary peaks, identified in alignment with the findings of other prominent researchers, emphasizes the potential existence of additional compounds or isomeric forms, further enriching our understanding of RNA modifications.

## 5. Conclusion

This study demonstrated the applicability of HPLC in the separation and identification of nucleosides and modified nucleosides. The obtained retention times serve as valuable reference points for future analyses, aiding in the quantification and characterization of these fundamental components. The presence of co-elution peaks emphasizes the intricate nature of nucleoside samples, warranting deeper investigations to unravel potential impurities or modifications. Ultimately, this research contributes to the understanding of nucleoside composition and complexity in biological systems.

## 6. Acknowledgments

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## 7. Conflict of Interest

The authors declare no conflicts of interest related to this research.

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