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# Hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents for the separation of nucleosides and nucleotide mono-, di- and triphosphates

María Mateos-Vivas, Encarnación Rodríguez-Gonzalo*, Diego García-Gómez, Rita Carabias-Martínez

*Departamento de Química Analítica, Nutrición y Bromatología, Universidad de Salamanca, 37008 Salamanca, Spain*

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ABSTRACT

A fast and efficient method for the simultaneous separation of highly polar compounds, in this case nucleosides and nucleotide mono-, di- and triphosphates, using hydrophilic interaction chromatography coupled with tandem mass spectrometry (HILIC–MS/MS) is proposed. This new separation method revealed the possibilities of the formation of hydrophilic ion-pairing compounds.

Three stationary phases (HILIC XBridge-Amide, HILIC-CoreShell and ZIC-HILIC) were assayed for the separation of 20 target analytes, and a detailed study of the composition of the mobile phase was made using different salts at different concentrations in a organic-rich mobile phase. We report that in order to prevent the adsorption of nucleotides on the LC–MS setup and to enhance their retention on the HILIC stationary phase, a mobile phase containing hexafluoro-2-propanol and different cations should be used. Four cations were evaluated: ammonium, diethylammonium, triethylammonium and tetrabutylammonium. The results revealed the formation of an ionic-association compound between the phosphorylated analytes and the cationic ion-pairing reagents, whose retention increased with the polarity of the cationic ion-pairing reagent.

HILIC XBridge-Amide was found to be the most suitable column for the separation of these analytes, and the optimized mobile phase consisted of an ACN/UHQ water mixture (3 min of isocratic elution using 82:18%, v/v and then a fast gradient from 18% to 22% of water) with 100 mM hexafluoro-2-propanol and 50 mM diethylamine ($_{w}^{w}$pH 9–$_{w}^{s}$pH 10). In a total analysis time of 8 min, good results were achieved in terms of resolution. Under these optimum conditions, a further comprehensive study of the retention mechanism was carried out.



## 1. Introduction

Nucleosides and nucleotides are a class of chemicals related to the regulation and modulation of several physiological processes [1], their determination being of considerable interest across a wide range of scientific areas such as biochemistry, medicine, genetics, metabolomics and food analysis [2,3]. Their concentration in cells provides information for understanding cellular energy metabolism, and their quantification in plasma samples can be used to assess oxidative stress. They are also useful for obtaining information about different pathologies, since it is well known that the concentration of some nucleosides and nucleotides is related to the immune response in oncologic patients [4–6]. Nucleosides and nucleotides are also found in foodstuffs. Their occurrence in these matrices helps the absorption of iron and stimulates damage repair in the gut and also enhances the immune response [7].

Owing to the high-polarity of these compounds, capillary zone electrophoresis (CZE) [8,9] and micellar electrokinetic chromatography (MEKC) [10,11] have been the techniques used for their determination in urine samples. Our group [12,13] has also developed a method for the determination of nucleotide monophosphates in infant formulas and in human milk by means of CZE–MS. Several different types of chromatographic separation have also been proposed based on reversed-phase liquid chromatography [14], ion-pair liquid chromatography [15], ion-exchange liquid chromatography [16] and hydrophilic interaction liquid chromatography (HILIC) [17–19].

* Corresponding author.
*E-mail address:* erg@usal.es (E. Rodríguez-Gonzalo).

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It has been suggested that in the analysis of phosphorylated compounds (e.g. nucleotides mono- di- and tri-phosphates) by LC–MS these analytes may interact with specific parts of the LC–MS setup [20]. These interactions are produced through the adsorption of the analytes on silanol groups or through the complexing of metallic cations with their phosphorylated residues. This results in a decrease in the chromatographic signal and in peak tailing [21]. Different strategies have been proposed to prevent hydrogen bonding between the silanol groups on the inner-wall surface of the fused silica capillary and phosphorylated compounds, such as derivatization of the free silanol groups of the silica by endcapping [22], or deactivation of the fused silica tubing with dimethyldichlorosilane [21]. The interaction between phosphopeptides and metals (mainly iron from stainless steel or solvents) in HPLC–ESI-MS devices affects detection due to the formation of phosphopeptide–Fe(III) complexes [23]. In order to prevent this interaction, several strategies have been proposed, such as the substitution of stainless steel tubing by polyether ether ketone (PEEK), the use of a high-pH mobile phase [20], pre-treatment of the chromatographic system with phosphoric acid [24], and the use of mobile phases with carbonate anions [25] or chelating agents such as EDTA [23].

Although CE has been applied to the determination of nucleosides and nucleotides, to the best of our knowledge there is no CE method for the simultaneous separation of these compounds. Moreover, chromatographic methods have focused on the separation of nucleosides or nucleotides and the few methods that do allow simultaneous separation are limited by excessively long separation times [7,26–29]. It may be concluded that the simultaneous separation of these compounds is a highly complex issue owing to their chemical similarities, their high polarity, which limits their retention in reversed-phase chromatography, and also because of the adsorption of these phosphorylated compounds on the chromatographic system, resulting in a lack of sensitivity and precision.

The aim of this work was thus to solve these issues by developing a method that would allow the rapid and efficient chromatographic separation of a large number of nucleosides and nucleotides by means of HILIC coupled with tandem mass spectrometry. In order to achieve this, a detailed study of the variables affecting HILIC separation was carried out, involving three HILIC columns and organic-rich mobile phases in the presence of cationic ion-pairing reagents. The retention mechanism in the final conditions was also characterized.

## 2. Experimental

### 2.1. Chemicals

Analytical standards of *adenosine* (A), CAS RN [58-61-7]; *cytidine* (C), CAS RN [65-46-3]; *guanosine* (G), CAS RN [118-00-3]; *inosine* (I), CAS RN [58-63-9]; *uridine* (U), CAS RN [58-96-8]; *adenosine 5′-monophosphate* (AMP), CAS RN [4578-31-8]; *cytidine 5′-monophosphate* (CMP), CAS RN [63-37-6]; *disodium salt hydrate of guanosine 5′-monophosphate* (GMP), CAS RN [5550-12-9]; *disodium salt of inosine 5′-monophosphate* (IMP), CAS RN [352195-40-5]; *disodium salt of uridine 5′-monophosphate* (UMP), CAS RN [3387-36-8]; *sodium salt of adenosine 5′-diphosphate* (ADP), CAS RN [20398-34-9]; *sodium salt hydrate of cytidine 5′-diphosphate* (CDP), CAS RN [63016-64-8]; *sodium salt of guanosine 5′-diphosphate* (GDP), CAS RN [43139-22-6]; *sodium salt of inosine 5′-diphosphate* (IDP), CAS RN [81012-88-6]; *disodium salt hydrate of uridine 5′-diphosphate* (UDP), CAS RN [27821-45-0]; *disodium salt hydrate of adenosine 5′-triphosphate* (ATP), CAS RN [34369-07-8]; *disodium salt of cytidine 5′-triphosphate* (CTP), CAS RN [36051-68-0]; *sodium salt hydrate of guanosine 5′-triphosphate* (GTP), CAS RN [36051-31-7]; *trisodium salt of inosine triphosphate* (ITP), CAS RN [35908-31-7] and *trisodium salt dihydrate of uridine 5′-triphosphate* (UTP), CAS RN [116295-90-0] were purchased from Sigma–Aldrich (Steinheim, Germany). The physical properties and the MS/MS parameters of these analytes are shown in Table S1.

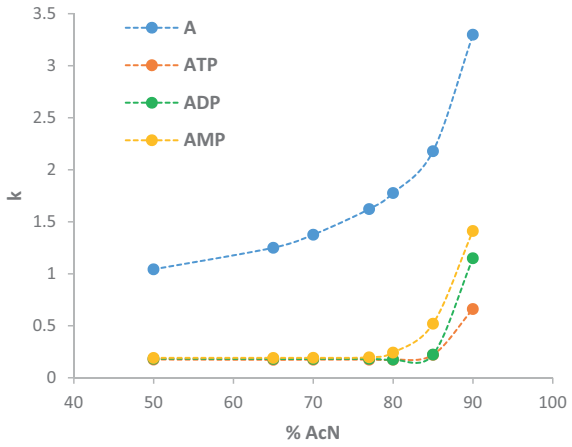


**Fig. 1.** Retention factors ($k$) for A, AMP, ADP and ATP, plotted versus the ACN percentage in the mobile phase, using the HILIC-CoreShell column and a mobile phase composed of only ACN and UHQ water.

The organic solvent, acetonitrile (ACN), was of HPLC grade (Merck, Darmstadt, Germany) and was used as received. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Noain, Spain) Ultramatic water purification system. The reagents used in the study of the mobile phase composition – hexafluoro-2-propanol (HFIP), diethylamine (DEA), triethylamine (TEA), tetrabutylammonium hydroxide (TBA), formic acid (HCOOH) and trifluoroacetic acid (TFA) – were provided by Sigma–Aldrich; reagent grade ammonia ($NH_3$) was purchased from Scharlau (Barcelona, Spain) and perchloric acid ($HClO_4$) was provided by Panreac (Barcelona, Spain).

### 2.2. Instrumentation

HPLC analyses were performed on an HP 1200 Series chromatograph from Agilent (Waldbronn, Germany). The 6410 Triple Quad mass spectrometer (Agilent, Waldbronn, Germany) was equipped with an electrospray ionization (ESI) source. The ESI settings were a capillary voltage of ±3500 V, a drying-gas flow of 12 L min$^{-1}$ at a temperature of 350 °C, and a nebulizer pressure of 35 psi. The analytical columns were a HILIC XBridge Amide packed with 3.5-μm particles from Waters (Milford, MA, USA), a HILIC-CoreShell packed with 2.6-μm particles from Phenomenex (Torrance, CA, USA), and a ZIC-HILIC packed with 3.5-μm particles from Merck (Darmstadt, Germany). The characteristics of the chromatographic columns used are shown in Table S2. $_w^wpH$ and $_w^spH$ values were determined with a Metrohm 691 pH Meter (Barcelona, Spain). Other mass spectrometry conditions were a fragmentor voltage of 100 V, collision energies optimized for each analyte and ranging from 5 to 10 eV for NUs to 25 eV for NMPs, NDPs, and NTPs; and a window of 1u for the isolation of the precursor ions, widened to 4u for the product ions. The dwell time for the analysis of the 20 analytes was 200 ms.

### 2.3. Preparation of standards

Initial stock solutions at 500 μg mL$^{-1}$ were prepared in UHQ water. These stock solutions were stored at 4 °C in brown glass bottles. Working solutions were prepared daily at 5 μg mL$^{-1}$ by mixing the appropriate amounts of the stock solution/s and diluting in ACN.