Quantitation of Nucleotides and Nucleosides without derivatization using Capillary Electrophoresis Coupled to Mass Spectrometry (CESI-MS)

CESI-MS Method for Analysis of Challenging Small Molecule Metabolites and Similar Drug Products

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Introduction

CESI-MS is the integration of capillary electrophoresis (CE) with electrospray ionization (ESI), into a single dynamic process within the same device (Figure 1). Operating at ultra-low flow rates, it helps reduce ion suppression and increases assay sensitivity for optimal results. Nucleotides are charged molecules, precursors and break-down products of DNA and RNA that also form the basic structure of cofactors. They act as energy carriers and mediators of important cellular processes[1]. Nucleosides are neutral and are also vital cellular components that inside the cell are metabolized by nucleokinases into the active nucleotide mono-, di-, triphosphates[2]. Nucleosides analogues are used as therapeutics of the treatment of viral infections, such as the human immunodeficiency virus (HIV)[1]. These polar compounds are usually difficult to analyze by traditional chromatography techniques, needing an extra derivatization step. This technical note describes an alternative workflow using CESI-MS for the quantitative analysis of selected nucleotides and nucleosides without derivatization with reproducibility and detection at nM levels. Application of this CESI-MS method is promising for analysis of these types of molecules in the pharmaceutical and biological (e.g. metabolomics, neurosciences) fields among others.

Advantages of CESI-MS Method

- No derivatization step
- Short analysis time
- Sensitive, quantitative method

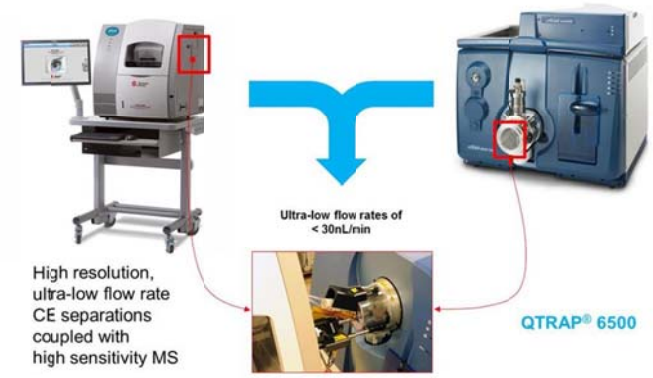


Figure 1. CESI-MS: CE integrated with QTRAP® 6500 MS

Experimental Procedure

Reagents:

- Ammonium acetate (AA), formic acid (FA) and, acetic acid were purchased from Sigma-Aldrich and used without any further purification. Ultrapure water (Type 1) was obtained from a Milli-Q Direct Ultrapure Water System. cytidine 5′-triphosphate (CTP), cytidine 5′-diphosphate (CDP), cytidine 5'-monophosphate (CMP), adenosine triphosphate (ATP), guanosine triphosphate (GTP), uridine triphosphate (UTP), thymidine triphosphate (TTP), rridine (Ur) and thymidine (Th) were purchased from Sigma-Aldrich.

Sample Preparation:

- Stock solutions of standard compounds were prepared in DDI water. Standard solutions, containing a mixture of the analytes, in the range of 0.25, 0.50, 1, 5, 10, 25 and 50 nM were also prepared in DDI water.

Instrumentation:

- **Capillary Electrophoresis (CE):** Separations were performed using a CESI 8000 Plus from SCIEX. The instrument was controlled using 32 Karat™ software v10.
- **Capillary:** A bare fused-silica capillary was used for the separation; 30 μm i.d. x 91 cm total length.
- **Mass Spectrometry (MS):** A QTRAP® 6500 mass spectrometer (SCIEX) coupled to the CESI through the CESI-MS Sprayer interface (see Figure 2) operating in positive ion mode with Analyst 1.7® software.

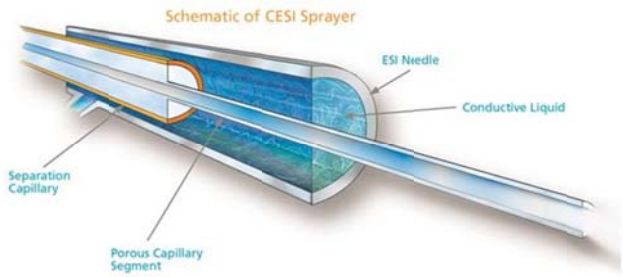


Figure 2: CESI Sprayer Interface

Instrument Conditions:

- **CESI:** Several background electrolytes (BGE) are recommended in the literature[1]. Ammonium acetate was selected because it was demonstrated in positive ionization mode. Several experimental conditions were tested to achieve the best separation using adenosine triphosphate (ATP) as model analyte. Formic acid was used as stacking agent. The final experimental conditions were:

  Background electrolyte: 12.5 mM Ammonium acetate pH 9.7.

  Stacking solution: 1% Formic acid

  Separation voltage: 30 kV with 2 psi of assisted pressure

  Sample storage temperature: 10 ºC

  Capillary temperature: 20 ºC

Instrument Conditions:

- **MS:** The QTRAP® 6500 mass spectrometer was run with electrospray ionization in positive ion mode and acquisition was from 100 to 600 *m/z* values. Conditions for Multiple Reaction Monitoring (MRM) were evaluated individually for each compound to select precursor and fragment ions. Table 1 provides the transitions that were used for the determination.

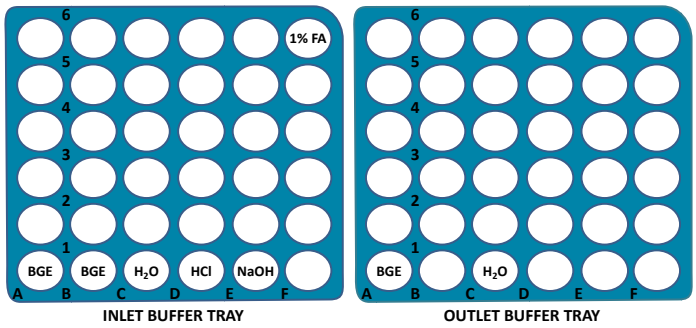
Name	Formula	Monoisotopic mass	*m/z* precursor ion	*m/z* fragment ion	Lowest detected concentration (nM)
Cytidine 5′-triphosphate (CTP)	$C_9H_{16}N_3O_{14}P_3$	482.9840	483.9	112.1	0.3
Cytidine 5′-diphosphate (CDP)	$C_9H_{15}N_3O_{11}P_2$	403.0182	404.0	112.1	0.3
Cytidine 5′-monophosphate (CMP)	$C_9H_{14}N_3O_8P_1$	323.0513	324.0	112.1	1.0
Adenosine triphosphate (ATP)	$C_{10}H_{16}N_5O_{13}P_3$	506.9952	507.9	136.1	0.3
Guanosine triphosphate (GTP)	$C_{10}H_{16}N_5O_{14}P_3$	522.9901	523.9	152.1	0.5
Uridine triphosphate (UTP)	$C_9H_{15}N_2O_{15}P_3$	483.9680	484.9	97.0	0.5
Thymidine triphosphate (TTP)	$C_{10}H_{17}N_2O_{14}P_3$	481.9887	482.9	81.0	0.5
Uridine (Uri)	$C_9H_{12}N_2O_6$	244.0690	245.1	113.0	5.0
Thymidine (Thy)	$C_{10}H_{14}N_2O_5$	242.0897	243.1	127.1	5.0

Table 1. MRM transitions for the analyzed compounds and the lowest detected concentrations (nM) for each one.

Data Analysis

- Analyses of the mass spectra were carried out using Peak View® software (SCIEX, Framingham, MA). The quantitative data analysis was performed using MultiQuant™ software (SCIEX, Framingham, MA).

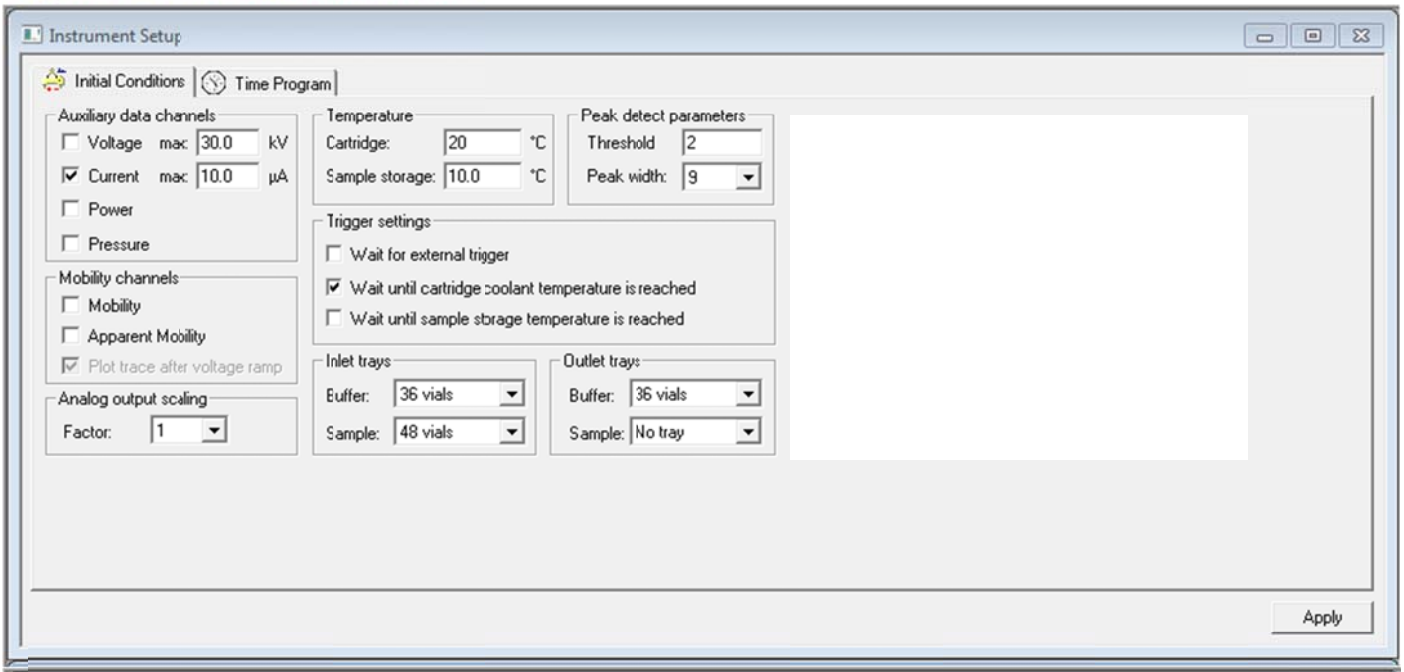
Method parameters: See Figures 3 and 4.

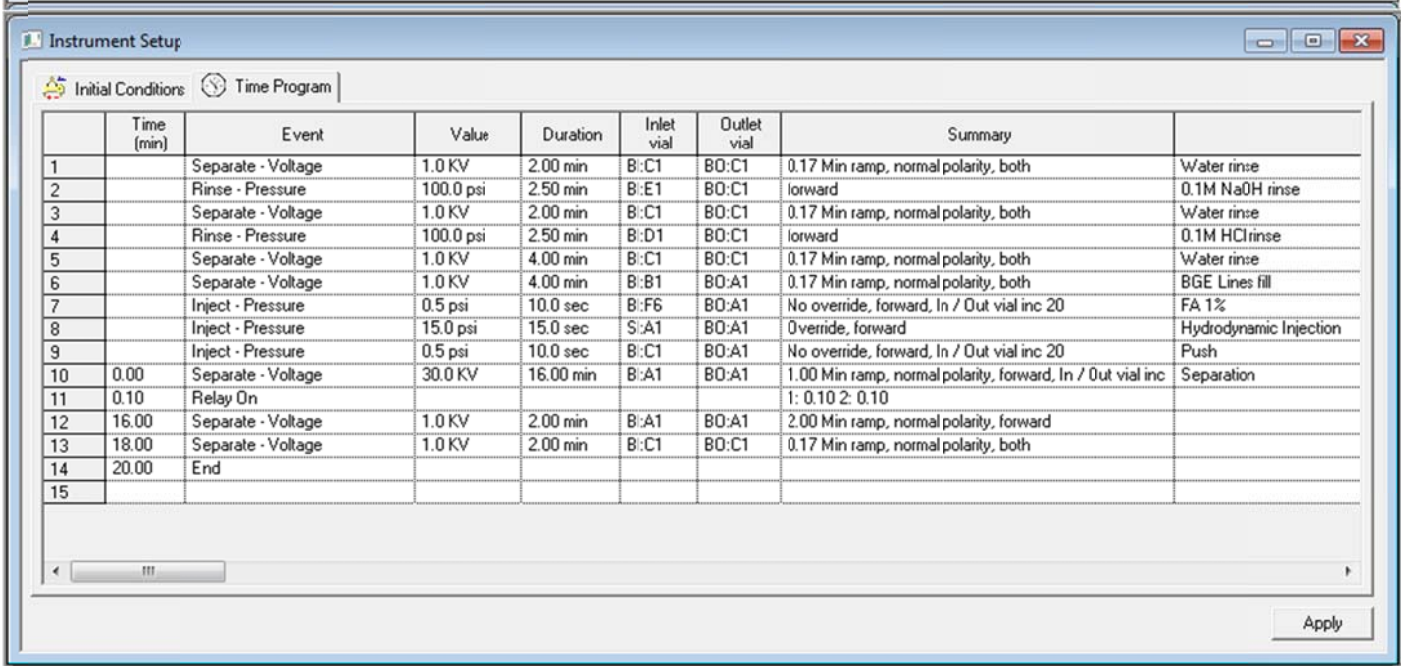


BGE: 12.5 mM Ammonium acetatte, pH 9.7

Figure 3: Scheme of the inlet and outlet buffer vial tray set-up for the CESI separation.

Separation Method Parameters





	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	
1		Separate - Voltage	1.0 KV	2.00 min	B:C1	BO:C1	0.17 Min ramp, normal polarity, both	Water rinse
2		Rinse - Pressure	100.0 psi	2.50 min	B:E1	BO:C1	forward	0.1M NaOH rinse
3		Separate - Voltage	1.0 KV	2.00 min	B:C1	BO:C1	0.17 Min ramp, normal polarity, both	Water rinse
4		Rinse - Pressure	100.0 psi	2.50 min	B:D1	BO:C1	forward	0.1M HCl rinse
5		Separate - Voltage	1.0 KV	4.00 min	B:C1	BO:C1	0.17 Min ramp, normal polarity, both	Water rinse
6		Separate - Voltage	1.0 KV	4.00 min	B:B1	BO:A1	0.17 Min ramp, normal polarity, both	BGE Lines fill
7		Inject - Pressure	0.5 psi	10.0 sec	B:F6	BO:A1	No override, forward, In / Out vial inc 20	FA 1%
8		Inject - Pressure	15.0 psi	15.0 sec	S:A1	BO:A1	Override, forward	Hydrodynamic Injection
9		Inject - Pressure	0.5 psi	10.0 sec	B:C1	BO:A1	No override, forward, In / Out vial inc 20	Push
10	0.00	Separate - Voltage	30.0 KV	16.00 min	B:A1	BO:A1	1.00 Min ramp, normal polarity, forward, In / Out vial inc	Separation
11	0.10	Relay On					1: 0.10 2: 0.10	
12	16.00	Separate - Voltage	1.0 KV	2.00 min	B:A1	BO:A1	2.00 Min ramp, normal polarity, forward	
13	18.00	Separate - Voltage	1.0 KV	2.00 min	B:C1	BO:C1	0.17 Min ramp, normal polarity, both	
14	20.00	End						
15								

Figure 4: Separation Method Parameters. In step 10 and 12, use 2 psi of forward pressure. In steps 3, 5, 6 and 13 a low separation voltage is applied with 100 psi in both directions to rinse in parallel the capillary in the separation and conductive lines.

Results and Discussion

- Analytical parameters such as peak shape and migration times were evaluated by injecting samples individually. A standard mixture was also injected to check separation performance (Figure 5).
- All compounds showed migration times below 15 min with high reproducibility and sharp peak shapes (Figure 5).
- Linearity and other parameters were evaluated using analyte sample mixtures from 0.25 – 50 nM for each compound.
- Figure 6 shows extracted electropherograms for the lowest concentration detected for the individual compounds, ranging from 0.25 to 1 nM for nucleotides and 5 nM for nucleosides (see Table 1).
- Calibration curves were obtained using MultiQuant™ software (SCIEX, Framingham, MA). The analytical method exhibited a quantitative linear response with r > 0.9 (Figure 7).

Conclusions

- A method for separation of some nucleotides and nucleosides was developed for CESI-MS with a short analysis time and minimum required sample volume. In addition this separation does not require derivatization of the analytes.
- Separation of challenging charged molecules: cytidine phosphates, ATP, GTP, UTP, TTP and nucleosides (Ur and Th) has been demonstrated using the CESI-MS system.
- CESI-MS could be applied for quantitative analysis with low detected concentrations (< 5 nM) for the analyzed compounds.
- Application of this CESI method is promising for metabolomics, biotransformation pharmacology, and analytical characterization studies where nucleotide/nucleoside-type molecules are quantitatively analyzed, particularly when samples are obtained with low volumes and/or and concentrations.

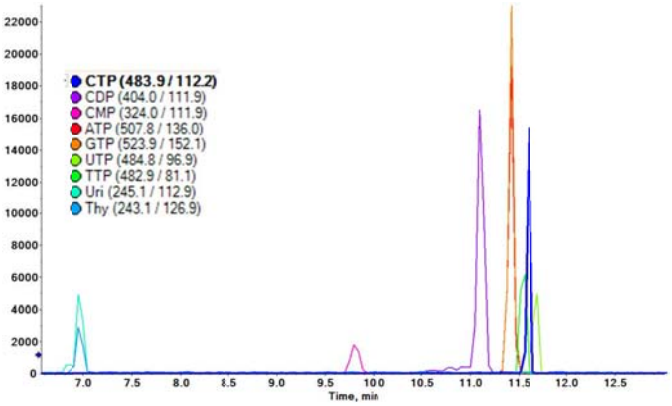


Figure 5: Extracted Ion Electropherograms (XIEs) of the analyzed compounds. All injected in the same experiment at 10 nM (see Table 1).

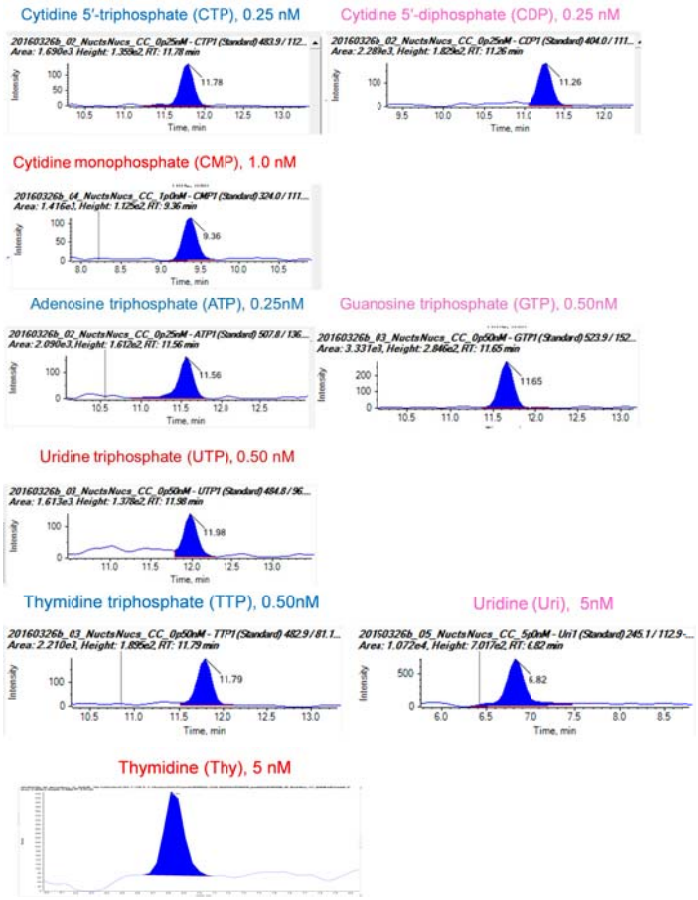


Figure 6: Extracted Ion Electropherograms (XIEs) for each of the analyzed compounds at the lowest concentration detected.

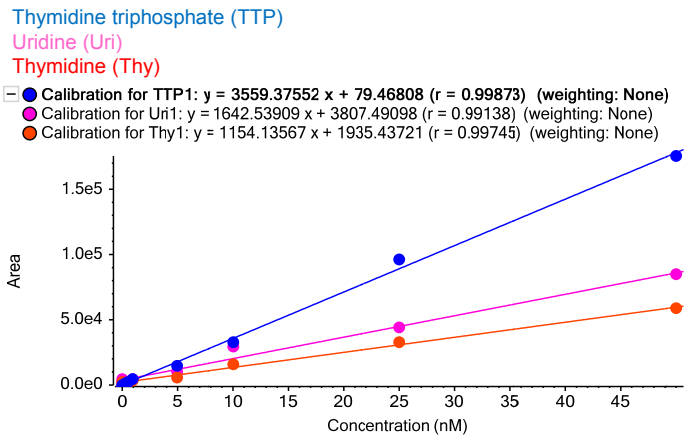


Figure 7: Calibration curves for the analyzed compounds. All analyte concentrations are ranging from 0 to 50 nM.

References

1. Willems, An V., Dieter L. Deforce, Carlos H. Van Peteghem, and Jan F. Van Bocxlaer. "Analysis of nucleic acid constituents by on-line capillary electrophoresis-mass spectrometry." *Electrophoresis* 26, no. 7-8 (2005): 1221-1253.

2. Liu, Charles C., et al. "Capillary electrophoresis-electrospray-mass spectrometry of nucleosides and nucleotides: Application to phosphorylation studies of anti-human immunodeficiency virus nucleosides in a human hepatoma cell line." *Electrophoresis* 26.7-8 (2005): 1424-1431.

Document number: RUO-MKT-02-2335-A

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