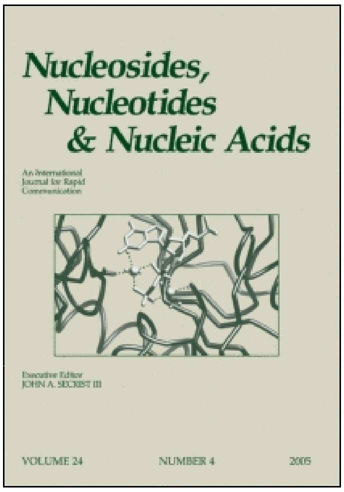

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MASS SPECTROMETRY BASED METHODS FOR ANALYSIS OF NUCLEOSIDES AS ANTIVIRAL DRUGS AND POTENTIAL TUMOR BIOMARKERS

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□ *The intracellular analysis of the phosphorylated metabolites of some anti-HIV nucleosides by liquid chromatography or capillary electrophoresis coupled with tandem mass spectrometry (LC-MS/MS or CE-MS/MS) has been realized on human peripheral blood mononuclear cells (PBMC), with limit of quantitation (LOQ) that allow them to be quantitated intracellularly. We described also the analysis of modified urinary nucleosides as potential tumor biomarkers.*

Keywords Antiviral; HIV; nucleosides; LC-MS/MS; therapeutic drug monitoring

INTRODUCTION

Modified nucleosides form an important family of drugs and tumor biomarkers. On the one hand, the nucleoside reverse transcriptase inhibitors (NRTIs) are inactive prodrugs, which must be phosphorylated into their triphosphate forms (NRTI-TPs) for activity. Since the pharmacokinetics of NRTIs varies between individuals, monitoring of patient plasma drug concentrations is becoming common, and because the NRTI-TPs are the active form, their intracellular analysis may be more appropriate.[1–3] On the other hand, modified urinary nucleosides (such as pseudouridine, cytidine, methylcytidine, ...) could be potentially invaluable in early cancer diagnosis, as they reflect altered RNA turnovers, and effective in monitoring of some cancers (as for instance the colorectal cancer).[4,5] As part of our ongoing drug discovery program, we have developed sensitive analytical methods specialized to study the bioactive nucleosides and their metabolites in various matrices. The tandem mass spectrometry (MS/MS) was used as a

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unique detection technique for either liquid chromatography or capillary electrophoresis. The immense selectivity of this extraordinary technique stems from the high specificity of the MS/MS detector which will show a signal for an analytie only if its unique combination of molecular ion and characteristic fragment ion is detected.

RESULTS AND DISCUSSION

We first turn our attention to the direct analysis of antiviral triphosphorylated metabolites of zidovudine (AZTTP), didanosine (ddATP), and stavudine (d4TTP) by LC-ESI-MS/MS on a PGC column to achieve separation of isobars. The best selectivity was obtained on a Hypercarb porous graphitic carbon column (PGC, Thermo Electron, Shandon, UK) for an acetonitrile-ammonium hydrogenocarbonate buffer in an elution gradient mode. The influence of organic modifier composition and salt concentration was studied by LC-UV. The optimized separation procedure allowed the quantification of 30 nucleotides within a runtime of 12 minutes (Figure 1). PGC column retention increased when the number of phosphate groups in the analytes increased. This suggests that the interaction between negatively charged phosphate groups and the π-electrons of PGC is a dominant factor in achieving both retention and resolution. However, the hydrophobic interaction between nucleoside base and PGC appeared to be more important when hydroxyl group—in $2'$ and $3'$ position on sugar moiety—and the difference in retention between deoxyribonucleotides and ribonucleotides increased. Moreover, the competition between hydrophobic interactions and $\pi - \pi$ interaction change: for ribonucleotide, the elution order is mono, di and triphosphate metabolite, while for deoxy- or dideoxy-ribonucleotides, the elution order is tri, di, and monophosphate. Hydrophobic interactions are greater than $\pi - \pi$ interaction for deoxy- and dideoxyribonucleotides. The validation parameters such as accuracy, intra-day and inter-day precision and limits of quantitation were determined in LC-MS/MS in negative mode (Table 1). The determination coefficient (r^2) for each compound was higher than 0.99. Moreover, the F1-values for the linearity test were higher than the critical value and consequently the slope was significantly different from 0 for a 0.05 α-risk level. The "goodness-of-fit" F2-test also was performed. F2-values for the linearity test were higher than the critical value for a 0.05 α-risk level showing that the linear model is suitable. Thus, a simple analytical procedure by LC-ESI-MS/MS coupling, using the powerful and singular retention of PGC support allowed us a direct and validated quantitation of triphosphorylated metabolites of stavudine (d4TTP), didanosine (ddATP), zidovudine (AZTTP), and abacavir (CBVTP) on PBMC extracts, with LOQ that allow them to be quantitated intracellularly.

We then turn our attention to the analysis of some antiviral triphosphorylated nucleosides by capillary electrophoresis-electrospray tandem mass

TABLE 1 Precision, accuracy, and limits of quantification from the validation assay for the determination of nucleotides in PBMC by LC-MS/MS

Analyte	Intra-day repeatability (%)	Inter-day repeatability (%)	Accuracy (%)	LOQ (ppb)
ddATP	<3.5	<8.1	110.6	0.3
CBVTP	<4.9	<6.3	109.7	0.5
d4TTP	<5.1	<5.6	111.2	0.3
AZTTP	<4.6	<5.1	90.4	0.5

spectrometry,[6] carried out a P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) coupled to a Quattro Ultima (Micromass, Manchester, UK) through a dedicated interface; this requires: the use of a volatile buffer system to ensure compatibility with the mass spectrometer; the addition of a sheath liquid (μL/minute) to compensate the low CE flow rate (nL/minute) in order to get stable spray, the optimization of the composition of sheath liquid, the optimization of nebulization gas and sheath liquid flow rates to get stable spray; the optimization of MS capillary voltage and also the optimization of the relative position between CE capillary outlet and electronebulization electrode. Several CE/MS parameters (composition and flow rate of sheath liquid) have been optimized to enhance spray

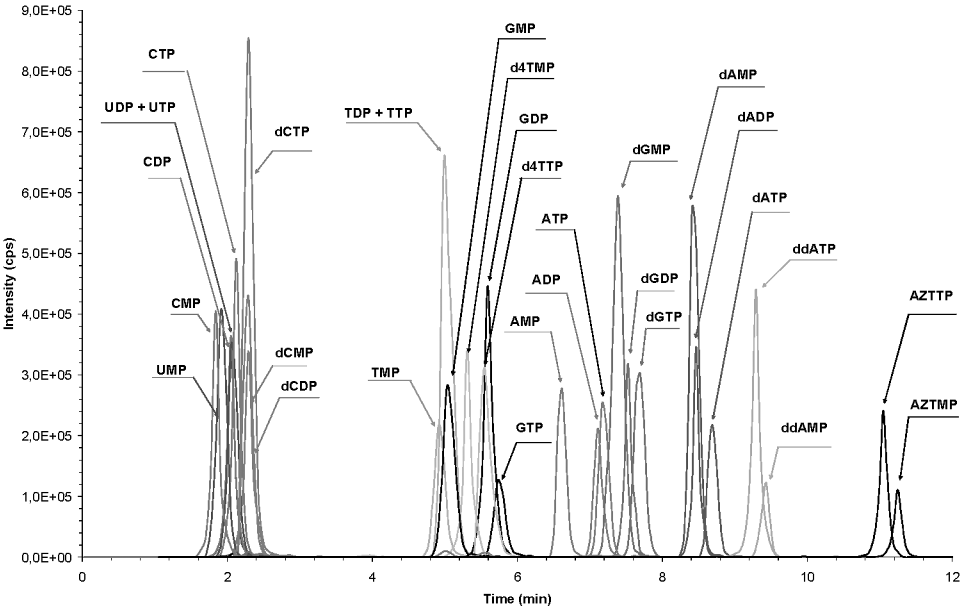

FIGURE 1 Analysis of 28 nucleotides including NRTI-TPs by LC-MS/MS. Operating conditions: LC: Hypercarb (Hypersil) 100 × 2.1 mm (5 μm), 25°C; flow: 0.2 mL/minute; injected vol.: 40 μL; mobile phase: (A) NH4HCO3 (I = 50 mM, pH 10) and (B) MeCN/acetone (70:30 v/v); gradient (%B): 10–50% in 15 minutes then 70% during 3 minutes; MS; capillary: −3.2 kV, 35 V, T: 360°C ; flow (gas nebulization): 40 L/minute; flow (desolvatation gas): 600 L/minute. Analyte concentration: 100 ng/mL.

TABLE 2 Comparison of LOQ of 7 urinary nucleosides between UV at 260 nm, MS, and MS/MS detections

LOQ (ppm)	U	G	C	5 mU	ddU	dA	3 mU
UV at 260 nm	1.7	1.3	1.6	1.3	0.9	3.5	1.4
MS	3.5	0.9	0.4	3.4	3.5	0.2	2.4
MS/MS	1.7	1.2	0.07	3.3	0.2	0.01	1.2

stability and MS sensitivity. Up to now, positive ionization mode has been performed, which achieve much more reproducible results than in negative mode. Finally, the determination of a pool of sixteen nucleotides, including anti-HIV mono- and triphosphated NRTIs, has been realized using a volatile buffer of *acetic acid/ammonia buffer (I = 40 mM, pH 10 and a sheat liquid based on 2,2,2-trifluoroethanol + 0,2 mM tridecafluoroheptanoic acid.* This method has been fully validated.[6]

More recently, we have developed the analysis of urinary potential tumor biomarkers by hydrophilic interaction chromatography (HILIC) LC-MS/MS. Selected urinary nucleosides including C_5-methyluridine, N_3-methyluridine, 2′,3′-dideoxyuridine (ddU) and 2′-deoxyadenosine (dA) were first extracted from urinary sample using a rapid and simple solid phase extraction (SPE, Envi-Carb 500 mg/6 mL, Supelco, Bellefonte, PA, USA). The HPLC procedure was optimised using several stationary phases. A conventional reversed phase column Waters (Milford, MA, USA) Atlantis C_{18} was assessed as reference of the chromatographic behaviour of the nucleosides. As a new separation mode in liquid chromatography three

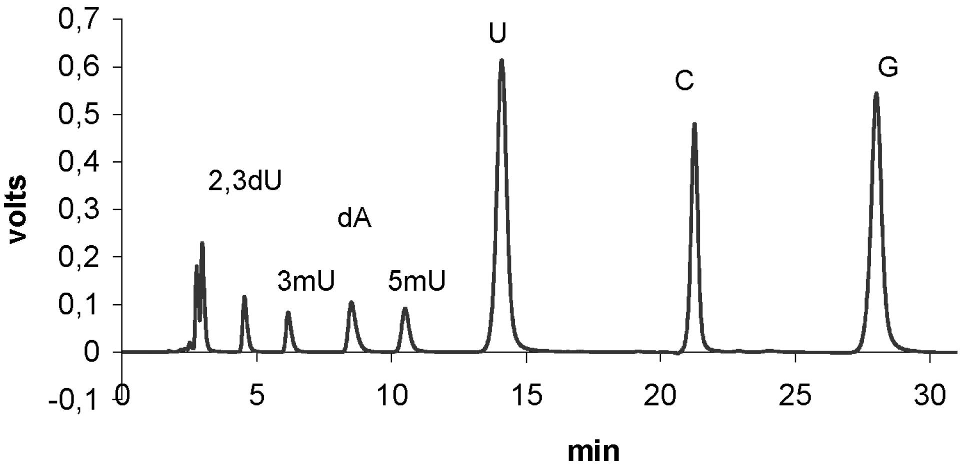

FIGURE 2 Analysis of 7 nucleosides including cancer markers by LC in HILIC mode. Operating conditions: LC: Astec apHera NH_2 100 × 2.1 mm (5 μm), 25°C; flow: 0.2 mL/minute; injected vol.: 20 μL; mobile phase: (A) Water and (B) MeCN gradient (%B): 90% during 10 minutes then 90–80% in 5 minutes then 80–70% during 15 minutes. UV at 260 nm.

HILIC columns (Waters Atlantis HILIC Silica, SeQuant (Umea, Sweden) ZIC-HILIC, Astec (Whippany, NJ, USA) apHera NH_2) were tested. It appears that the SeQuant ZIC-HILIC and the Astec apHera NH_2 columns allowed the separation of the selected nucleosides in less than 28 minutes, with a really good efficiency and without any salt required in the mobile phase (Figure 2). The optimized HPLC conditions were thus fully suitable for MS/MS coupling. Indeed, the high amount of organic solvent in the mobile phase in HILIC enhanced the MS signal allowing a 200 times increase of the S/N ratio for dA. The LC-MS/MS coupling was realised on a Perkin Elmer SCIEX API-300 (Forster City, CA, USA) in ESI+, using a MeCN-water gradient. The analysis of selected nucleosides was favoured in UV and MS/MS. In all case, an enhancement of sensitivity of MS/MS *vs* UV was observed for C, ddU and dA and not 5 mU, meanwhile the same sensitivity was observed for U, G, and 3 mU (Table 2). It is interesting to quote that the HILIC allow a specific and sensitive detection in MS/MS. This HILIC mode is also suitable for an on-line purification because elution strength of solvents is reversed between HILIC and reversed phase chromatography. The analysis of 26 nucleosides known as cancer biomarkers through an on-line cleaning LC-MS/MS method is on-going within our group.

REFERENCES

1. Fletcher, C.V.; Acosta, E.P.; Henry, K.; Page, L.M.; Gross, C.R.; Kawle, S.P.; Remmel, R.P.; Erice, A.; Balfour, H.H. *Clin. Pharm. Ther.* **1998**, 64, 331.
2. Pereira, A.S.; Tidwell, R.R. *J. Chromatogr. B* **2001**, 764, 327 (review).
3. Banoub, J.H.; Newton, R.P.; Esmans, E.; Ewing, D.F.; Mackenzie, G. *Chem. Rev.* **2005**, 105, 1869.
4. Xu, G.; Liebich, H.M. *Am. Clin. Lab.*, **2001**, 20, 22.
5. Jonhson, S. A. *Clin. Pharmacokinei.* **2000**, 39, 5 (review).
6. Bezy, V.; Chaimbault, P.; Morin, Ph.; Unger, S.E.; Bernard, M.-C.; Agrofoglio, L.A. *Electrophoresis* **2006**, 27, 2464.