Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 315 (2004) 151–159
BBRC
www.elsevier.com/locate/ybbrc

A new method for analysis of AZT-triphosphate and nucleotide-triphosphates

Jeroen J.A. van Kampen,[a] Pieter L.A. Fraaij,[a] Vishal Hira,[a] Annemarie M.C. van Rossum,[a] Nico G. Hartwig,[a] Ronald de Groot,[a] and Theo M. Luider[b,*]

[a] *Department of Paediatrics, Sophia Children's Hospital, Erasmus MC, Rotterdam, The Netherlands*
[b] *Laboratory of Neuro-Oncology, Department of Neurology, Erasmus MC, Rotterdam, The Netherlands*

Received 7 January 2004

Abstract

We have developed a new method based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for analysis of zidovudine-triphosphate and (deoxy)nucleotide-triphosphates, which ultimately can be used for nucleoside reverse transcriptase inhibitor (NRTI) treatment monitoring in HIV-1 infected children and adults. Four different matrices were compared for sensitivity and reproducibility of zidovudine-triphosphate detection and anthranilic acid mixed with nicotinic acid (AA/NA) was selected as most suitable matrix. Solutions of zidovudine-triphosphate, ATP, and dGTP were detected up to 0.5 fmol per sample. Furthermore, intracellular zidovudine-triphosphate, ATP, and dGTP were detected in peripheral blood mononuclear cells (PBMCs). Zidovudine-triphosphate, ATP, and dGTP yield identical mass spectra, however MALDI-TOF post-source decay analysis can be used for discrimination between these compounds. We conclude that this method based on MALDI-TOF MS can be used for analysis of intracellular zidovudine-triphosphate and (deoxy)nucleotide-triphosphates in PBMCs.

Keywords: HIV-1/AIDS; MALDI; NRTI; Nucleotide; Intracellular; Triphosphate; Children

Institution of optimal treatment of HIV-1 infected patients poses a major challenge. Treatment normally consists of a combination of three classes of antiretroviral drugs: the protease inhibitors (PIs), the non-nucleoside reverse transcriptase inhibitors (NNRTIs), and the nucleoside reverse transcriptase inhibitors (NRTIs). A major feature of these three drug classes is that large interindividual and intraindividual differences are observed in their pharmacokinetics [1–5]. This is even more important when one considers the relation between viral suppression and plasma concentration of PIs and NNRTIs [1,3,6–9]. Therefore, we routinely perform pharmacokinetic analyses of PIs and NNRTIs in HIV-1 infected children and adjust the dose of PIs and NNRTIs to maintain optimal plasma concentrations. This approach has resulted in favorable results with 69% viral response after 2 years of treatment [10].

However, such an approach is not possible for NRTIs. NRTI plasma concentrations correlate poorly with HIV-1 activity, since NRTIs are prodrugs that are intracellularly converted to active NRTI-triphosphates (NRTI-TPs) using the kinases from the host cell [11]. Intracellularly, the NRTI-TP competes with its corresponding endogenous deoxynucleotide-triphosphate (dNTP) for incorporation into viral DNA by HIV reverse transcriptase. Incorporation of NRTI-TP terminates elongation of viral DNA, thus preventing viral replication. In vitro studies with peripheral blood mononuclear cells (PBMCs) show that HIV-1 activity of NRTIs correlates more closely to the ratio of the intracellular NRTI-TP concentration and the corresponding intracellular dNTP concentration than to the intracellular NRTI-TP concentration alone [12–14]. In addition, a clinical study with HIV-1 infected adults shows that the intracellular NRTI-TP concentration alone correlates to HIV-1 activity [15].

* Corresponding author. Fax: +31-10-408-83-65.
E-mail address: t.luider@erasmusmc.nl (T.M. Luider).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.01.032

Current methods for quantification of NRTI-TPs, such as radio immunoassays, require large blood samples and do not allow high throughput analysis [16,17]. This complicates patient related studies, which require analysis of multiple blood samples of a large group of HIV-1 infected patients. Methods have been developed, such as HPLC coupled to electrospray ionization (ESI) MS, which allow for high throughput analysis of NRTI-TPs [18–22]. These methods still require at least 7 ml of blood to obtain sufficient material for analysis. This amount of blood allows for patient related studies in HIV-1 infected adults, but still complicates NRTI-TP studies in HIV-1 infected children, since such studies require multiple blood samples from one individual on a single day.

We aimed to develop a relatively easy method for analysis of intracellular NRTI-TP, which ultimately can be used for studies in HIV-1 infected children and adults. We studied the usability of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for this method, since: (1) MALDI-TOF MS methods have been developed for quantification of drugs, such as antibiotics and intracellular tetraphenylphosphonium [23–25]. (2) MALDI-TOF MS is able to detect compounds at very low concentrations, which allows for analysis when only small amounts of material can be obtained [26]. (3) MALDI-TOF MS is more tolerant to salts and other contaminants compared to other forms of MS [26]. This allows for reduction in sample preparation steps, which reduces sample loss and sample preparation time. (4) MALDI-TOF MS allows for rapid and fully automated analysis of large amounts of samples, which allows for high throughput use [27,28].

We studied the use of MALDI-TOF MS for analysis of the triphosphate form of the most widely used NRTI zidovudine (AZT-TP). We also examined if MALDI-TOF MS is capable of detecting dNTPs (dGTP), since in vitro studies show that HIV-1 activity correlates better to the ratio of intracellular NRTI-TP and dNTP concentration than to the intracellular NRTI-TP concentration alone [12–14]. In addition, we studied the analysis of NTPs (ATP, CTP, GTP, and UTP) by MALDI-TOF MS, since these compounds share many features (e.g., size, triphosphate group) with dNTPs and NRTI-TPs and could cause interferences. To our knowledge, this is the first time MALDI-TOF MS is used for analysis of NRTI-TP and endogenous (d)NTPs. Therefore, no suitable matrices are known for MALDI-TOF analysis of these compounds. We studied the usability of four different matrices, which are currently used in MALDI-TOF analysis of related compounds such as oligonucleotides. Subsequently, the most suitable matrix was used for analysis of intracellular AZT-TP in PBMCs. Finally, we studied the use of MALDI-TOF post-source decay (MALDI-PSD) analysis for discrimination of AZT-TP from ATP and dGTP, since these three compounds have the same molecular weight, which can cause interferences when using a method based on mass spectrometry.

Materials and methods

Standard nucleotide solutions. ATP (molecular weight 507.2 Da), CTP (483.1 Da), GTP (523.3 Da), dGTP (507.2 Da), UTP (484.1 Da) (Amersham, Sweden), and AZT-TP (507.2 Da) (Calbiochem, Germany) were diluted with HPLC-grade water and stored at −80 °C until analysis.

Preparation of matrix solutions. Solution I: 45 M anthranilic acid (AA) (Fluka, Switzerland) was mixed with 45 M nicotinic acid (NA) (Fluka, Switzerland) and 55 mM diammoniumhydrogencitrate (DAHC) (Fluka, Switzerland) in 45% acetonitrile (Aldrich, Germany). Solution II: 3-hydroxy picolinic acid (3-HPA) (Aldrich, Germany) was mixed with DAHC and HPLC-grade water, until the solution had a concentration of 3 mg/ml 3-HPA and 9 mg/ml DAHC. Solution III: 5-methoxy salicylic acid (5-MSA) was saturated in one part acetonitrile and one part DAHC mixed with HPLC-grade water (50 mM). Subsequently, saturated 5-MSA solution was 10 times diluted with DAHC mixed with HPLC-grade water (50 mM). Solution IV: 2,5-dihydroxybenzoic acid (2,5-DHB) was mixed with DAHC and HPLC-grade water, until the solution had a concentration of 3 mg/ml 2,5-DHB and 9 mg/ml DAHC. Fresh matrix solutions were prepared in Teflon tubes on the day of analysis.

Comparison of matrix solutions. Matrix solutions I–IV were compared for reproducibility and limit of detection of AZT-TP analysis in standard solution.

Relation between AZT-TP concentration and signal-to-noise ratio. Different AZT-TP dilutions were analyzed to assess the effect of the AZT-TP concentration on the signal-to-noise ratio. The signal-to-noise ratio (*S*/*N*) was calculated by dividing the maximal signal height to the local noise level. Samples were only measured if sample crystallization was observed by light microscopy (needle crystallization).

PBMC isolation. Venous blood from healthy volunteers was collected in Vacutainer CPT tubes (Becton–Dickinson). PBMCs were separated by centrifugation at 2000 rpm for 20 min and washed twice with PBS (1500 rpm for 10 min and 1200 rpm for 15 min, respectively). PBS was discarded and 3 ml growth media (RPMI supplemented with 10% heat-inactivated fetal calf serum, 10,000 IU/ml penicillin, 10,000 IU/ml streptomycin, and 20% DMSO) were added.

PBMC incubation with AZT. Growth media were added until a final concentration of 1×10^6 PBMCs/ml was reached. One microliter AZT solution (Fluka, Switzerland) was added to reach a final concentration of 10 μM. An equal amount of deionized water was added for the creation of "negative control" samples. Cultures were incubated at 37 °C for 3 h. After incubation, cells were centrifuged at 3800 rpm for 5 min and washed with PBS (3800 rpm for 5 min). "Positive controls" were created by adding 200 fmol AZT-TP to the PBMC pellet just before extraction.

Nucleotide extraction from PBMCs. To the PBMC pellet 500 μl cold MeOH (60%) was added and nucleotides were extracted at 4 °C overnight. After extraction, samples were centrifuged (10,000 rpm for 5 min), supernatants were collected and lyophilized with a SpeedVac (Savant, USA) for 45 min. Residues were stored at −80 °C until analysis. Per sample 0.85×10^6 PBMCs were used for intracellular AZT-TP analysis.

MALDI-TOF analysis. Matrix solution (0.5 μl) was pipetted onto an Anchor Chip target plate (Bruker Daltonics, Germany) and dried at room temperature. Subsequently 0.5 μl sample was pipetted onto the crystallized matrix and dried at room temperature. Analysis was performed by a BIFLEX III MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) using the negative reflectron mode. Laser attenuation was set at 40 (UNIX operating system). Hundred shots were used for each mass spectrum. Post-source decay (PSD) analysis was

performed on PBMC samples and on standard solutions of AZT-TP, ATP, and dGTP by the BIFLEX III MALDI-TOF mass spectrometer in the negative mode.

Prediction of fragmentation pattern of AZT-TP, ATP, and dGTP. MS Fragmenter software from ACD/Labs was used for prediction of the fragmentation pattern of AZT-TP, ATP, and dGTP.

Results and discussion

Comparison of different matrices for AZT-TP analysis

We tested four different matrices on their sensitivity and reproducibility of AZT-TP detection. Both the AA/NA (solution I) and 3-HPA (solution II) matrices were able to detect the expected mass signal of AZT-TP up to 0.5 fmol per sample (Figs. 1 and 2). The reproducibility of the mass spectra, however, is much better when AA/NA is used compared to the use of 3-HPA. The expected mass signal of AZT-TP was not detected when 25 fmol AZT-TP was analyzed with the 5-MSA matrix (solution III). The 2,5-DHB matrix (solution IV) itself yielded a signal at *m/z* of 505.3, which can interfere with the detection of AZT-TP. AA/NA is therefore the most suitable matrix for analysis of AZT-TP.

The effect of the AZT-TP concentration on the signal-to-noise ratio

Known quantities of AZT-TP were measured to analyze the relation with the signal-to-noise ratio. Fig. 3 shows a clear linear relation between the signal-to-noise ratio and AZT-TP concentration. This allows for rough estimation of the AZT-TP concentration. However, more exact values of the intracellular AZT-TP concentration are required for patient related research, which cannot be obtained by estimation based on the AZT-TP concentration versus signal-to-noise ratio curve. There are more accurate ways for quantification by MALDI-TOF MS. By adding a known concentration of a compound (internal standard) to the sample, one can compare signal-to-noise ratio of the analyte of interest with the signal-to-noise ratio of the internal standard. This method has resulted in an accurate quantification of different compounds, as published earlier [22–24].

Detection of (d)NTPs in standard solution

dGTP was used for exploring the usability of MALDI-TOF MS for dNTP analysis. The expected mass signal of dGTP (*m/z* 506.2) was detected up to a dilution of 0.5 fmol per sample when using the AA/NA matrix. This shows that MALDI-TOF MS is able to detect dNTP, which is necessary for exploring its ability of measuring the ratio of intracellular NRTI-TP and corresponding dNTP in PBMCs. It is likely that other dNTPs, such as dTTP, can be detected by MALDI-TOF MS, since related compounds (NTPs, dGTP, and AZT-TP) can be detected up to 0.5 fmol per sample. ATP, CTP, GTP, and UTP were used for exploring the usability of MALDI-TOF MS for NTP analysis. The expected mass signal of ATP (*m/z* 506.2) was detected up to a dilution of 0.5 fmol per sample when using the AA/NA matrix. Samples of 500 and 50 pmol of ATP, CTP, GTP, and UTP were analyzed using the 3-HPA matrix.

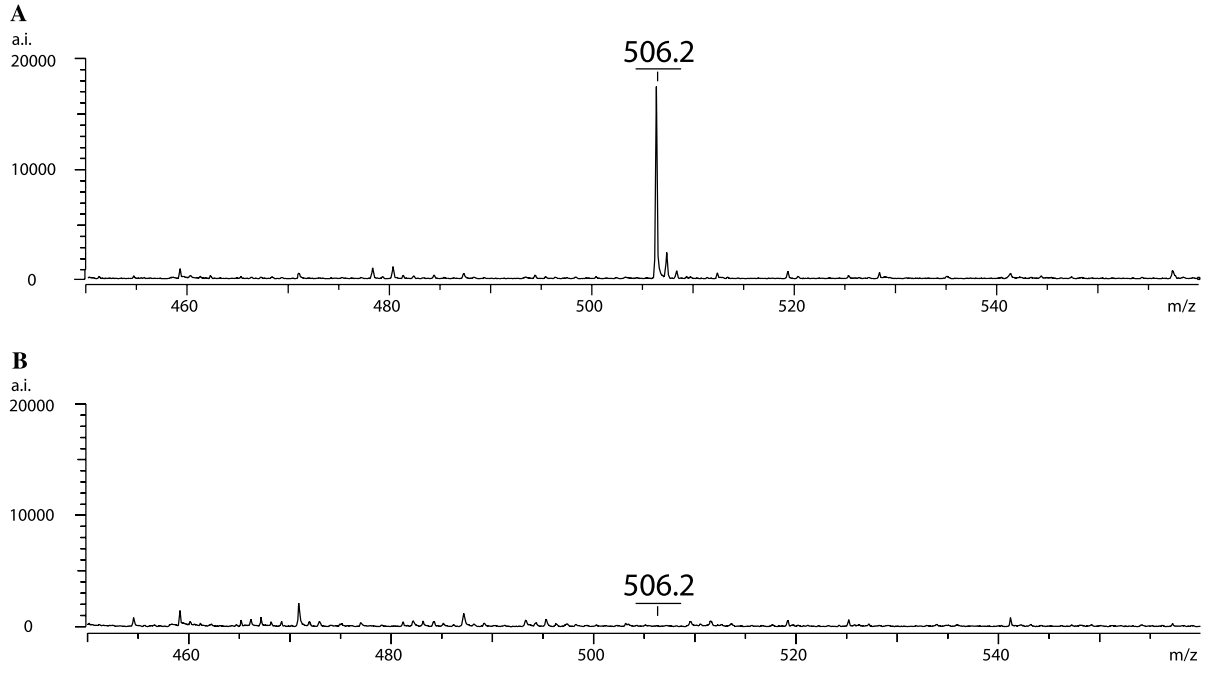

Fig. 1. Measurement of 500 fmol AZT-TP (A). An expected signal was found at *m/z* 506.2. The negative control sample (B) yielded no signal at *m/z* 506.2. AA/NA was used for matrix. a.i., absolute intensity. *m/z*, mass-to-charge ratio.

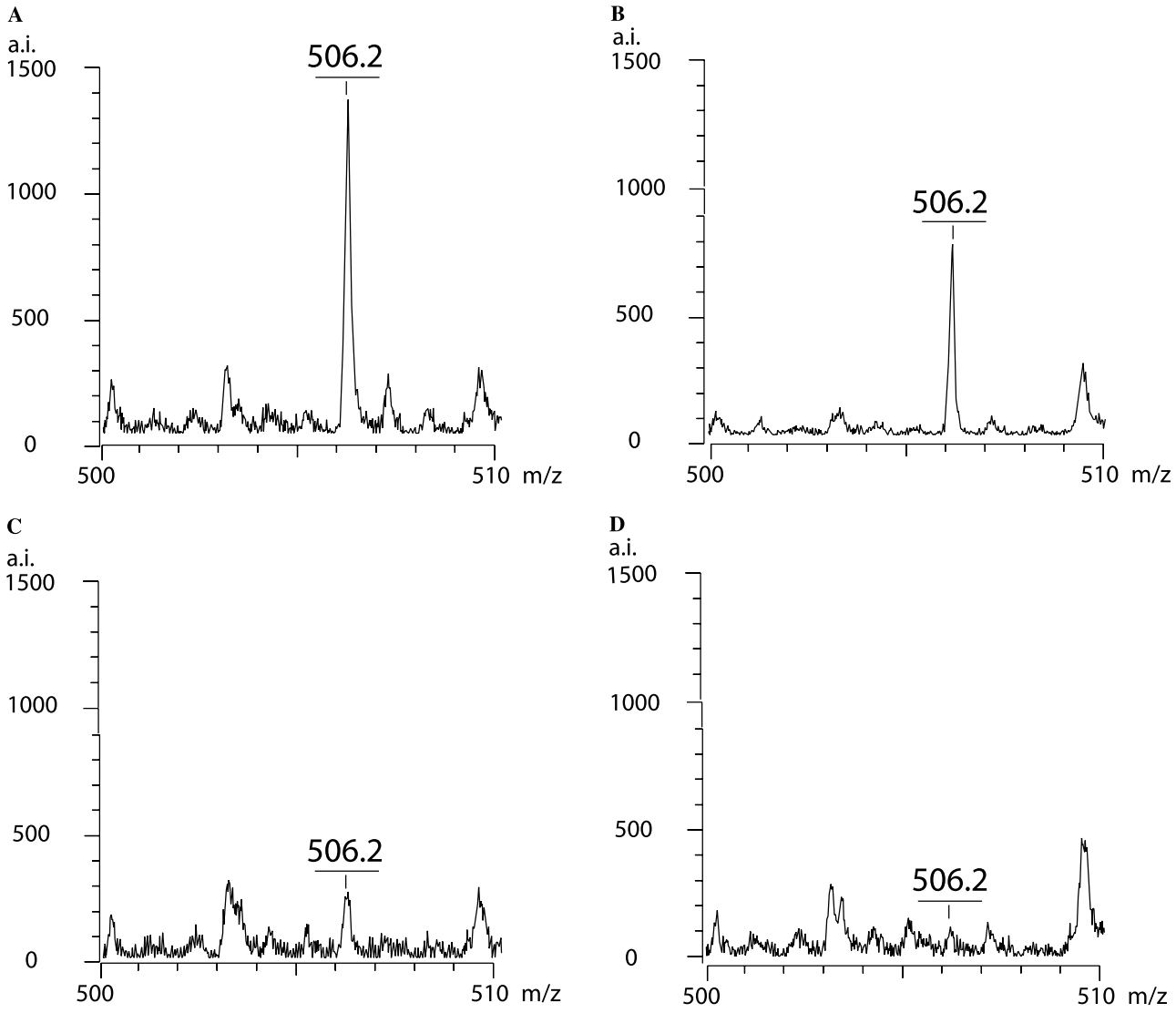

Fig. 2. Measurement of 5.0 fmol (A), 2.5 fmol (B), and 0.5 fmol (C) AZT-TP. An expected signal was found at *m/z* 506.2 when AZT-TP was diluted up to 0.5 fmol per sample. The negative control sample (D) yielded no signal at *m/z* 506.2. AA/NA was used for matrix.

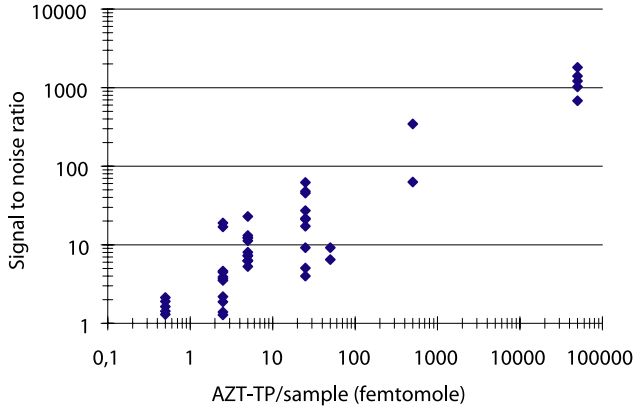

Fig. 3. A linear relation was found between AZT-TP concentration and signal-to-noise ratio. AA/NA was used for matrix.

All mass signals were detected at the expected mass-to-charge ratios (*m/z* 506.2, *m/z* 482.1, *m/z* 522.3, and *m/z* 483.1, respectively). This shows that MALDI-TOF MS is able to detect NTP, which is necessary for predicting possible interference of such compounds when analyzing AZT-TP. ATP, dGTP, and AZT-TP yield a mass signal at the same mass-to-charge ratio (*m/z* 506.2) in standard nucleotide solutions. This complicates future experiments for AZT-TP quantification. Therefore, we studied if ATP and dGTP also interfered with the detection of AZT-TP in PBMCs.

Intracellular AZT-TP in PBMCs

The analysis of the extract of PBMCs incubated with AZT revealed a mass signal at the expected *m/z* of 506.2 (Fig. 4). This expected mass signal was again detected

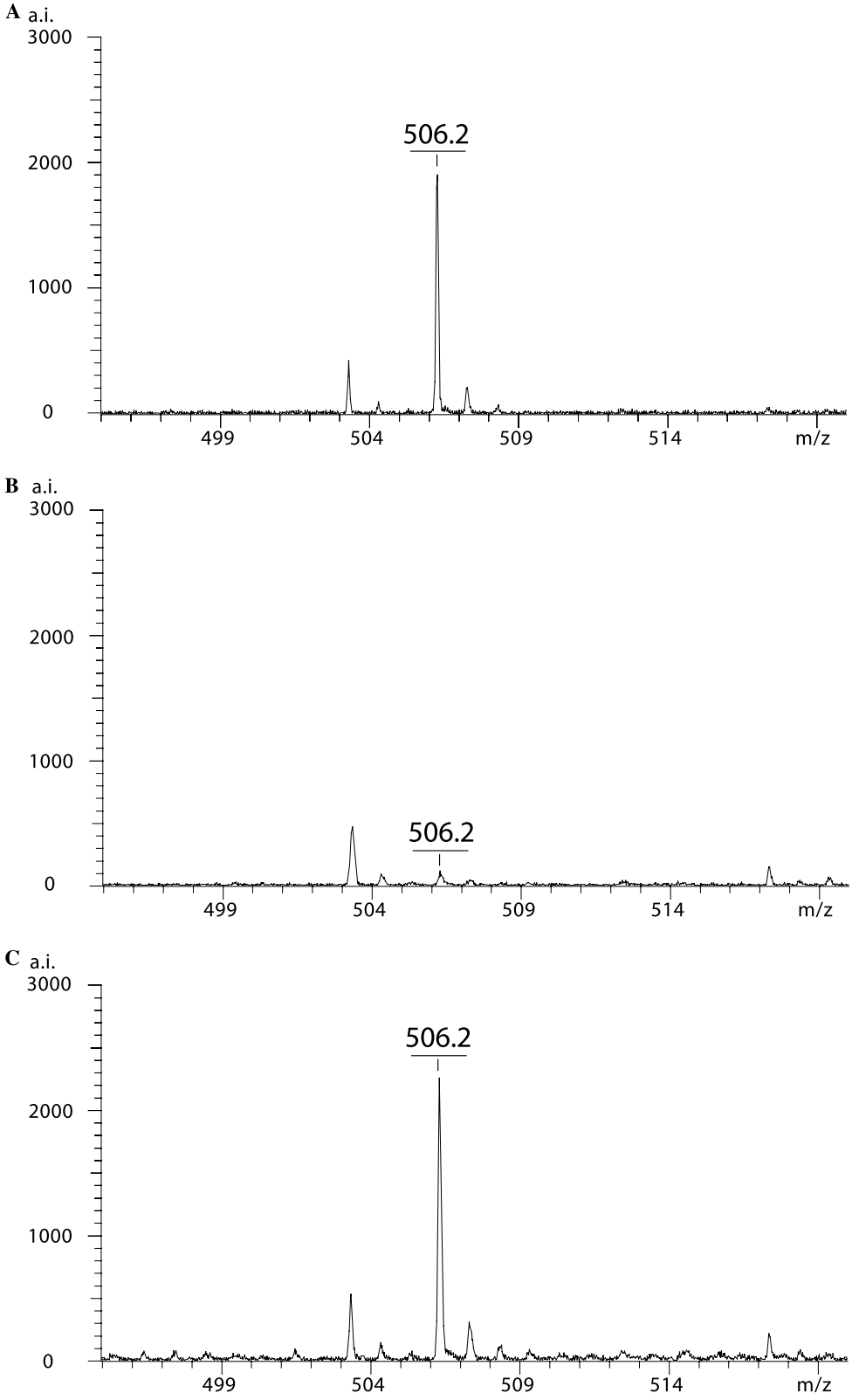

Fig. 4. Mass signals of intracellular ATP, dGTP, and AZT-TP were detected at the expected *m*/*z* 506.2 in PBMCs incubated with AZT (A). Mass signals of intracellular ATP and dGTP were detected at the expected *m*/*z* 506.2 in PBMCs not incubated with AZT (negative control) (B). Mass signals of intracellular ATP, dGTP, and AZT-TP are found at the expected *m*/*z* 506.2 in PBMCs not incubated with AZT and spiked with 200 fmol AZT-TP (positive control) (C). Per sample 0.85×10^6 PBMCs were used. AA/NA was used for matrix.

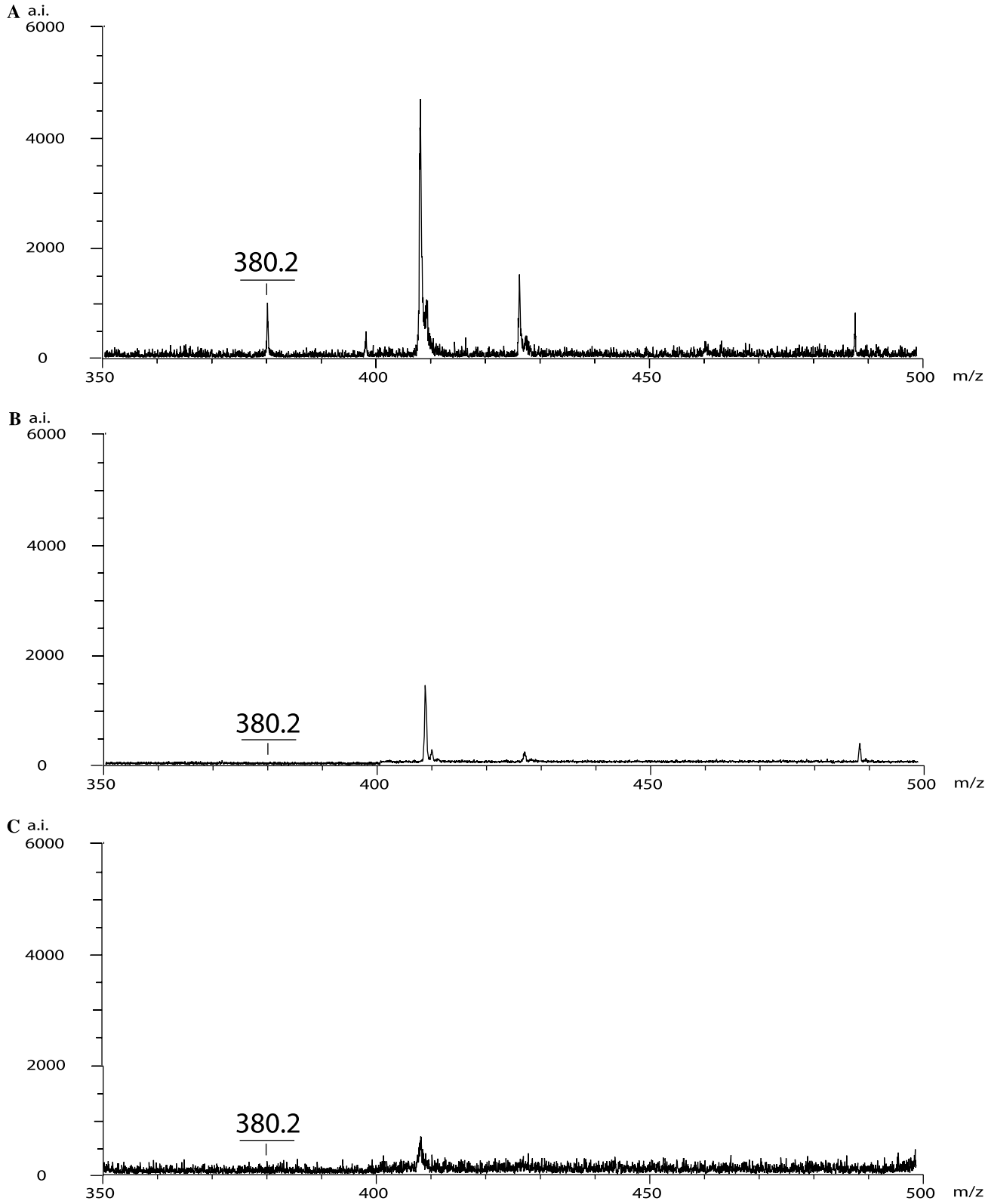

Fig. 5. MALDI-PSD analysis of 0.5 fmol AZT-TP revealed a unique mass signal at *m/z* 380.2 (A). This mass signal was not found by MALDI-PSD analysis of 0.5 nmol ATP (B) and dGTP (C). AA/NA was used for matrix.

when 200 fmol AZT-TP was added to the pellet of PBMCs not incubated with AZT (positive control). The extract of PBMCs not incubated with AZT (negative control) also yielded a mass signal at *m/z* of 506.2. This shows that AZT-TP, ATP, and dGTP can be detected in PBMCs by MALDI-TOF. However, ATP and dGTP

interfere with AZT-TP detection in PBMCs. It is not possible to estimate the AZT-TP concentration by comparing signal-to-noise ratio of the mass signal at *m/z* 506.2 in negative controls and in PBMCs incubated with AZT. AZT affects the nucleotide pool size, thus we cannot assume that ATP and dGTP are present in the same concentrations in negative controls and in PBMCs incubated with AZT [29]. Therefore, we have searched for a method to discriminate AZT-TP from ATP and dGTP.

Software prediction of AZT-TP, ATP, and dGTP fragmentation

Molecules can be fragmented and the mass of these fragments can be measured by MALDI-PSD analysis. The software prediction of the fragmentation pattern of AZT-TP, ATP, and dGTP revealed a unique fragment for AZT-TP. This fragment has a *m/z* of 382 (381 Da + 1 Da), when measuring in the positive mode. Since we measure in the negative mode, a proton is lost and the fragment should be detected at *m/z* 380 (381 Da – 1 Da).

Fragmentation of AZT-TP, ATP, and dGTP in standard nucleotide solutions

MALDI-PSD analysis was used for detection of the unique AZT-TP fragment. As predicted by software, the MALDI-PSD analysis of AZT-TP, ATP, and dGTP in standard solution revealed a unique mass signal for AZT-TP at *m/z* 380.2 (Fig. 5). This signal was not observed when analyzing ATP and dGTP by MALDI-PSD. Subsequently, we explored the ability of MALDI-PSD analysis for detecting the AZT-TP fragment in PBMCs.

Fragmentation of AZT-TP present in PBMCs

Fig. 6 depicts the detection of the mass signal at the expected *m/z* 380.2 in PBMCs incubated with AZT. This signal was not observed in the analysis of PBMCs not incubated with AZT. Thus, MALDI-PSD analysis is able to discriminate AZT-TP from ATP and dGTP in standard nucleotide solutions and in PBMCs.

The development of a method for quantification of NRTI-TP in HIV-1 infected children requires that the analysis can be performed on small amounts of PBMCs. Approximately one million PBMCs can be derived from one ml blood, which is an acceptable blood sample size for studies in HIV-1 infected children. Font et al. [21] showed that the intracellular AZT-TP concentration ranges from 38 to 193 fmol per million PBMCs in HIV-1 infected adults. The signal-to-noise ratio of AZT-TP detection in standard solution obtained by our method is larger than 10 in this range of measurement. However, the limit of detection of the unique AZT-TP fragment in PBMCs is probably higher than the limit of detection of

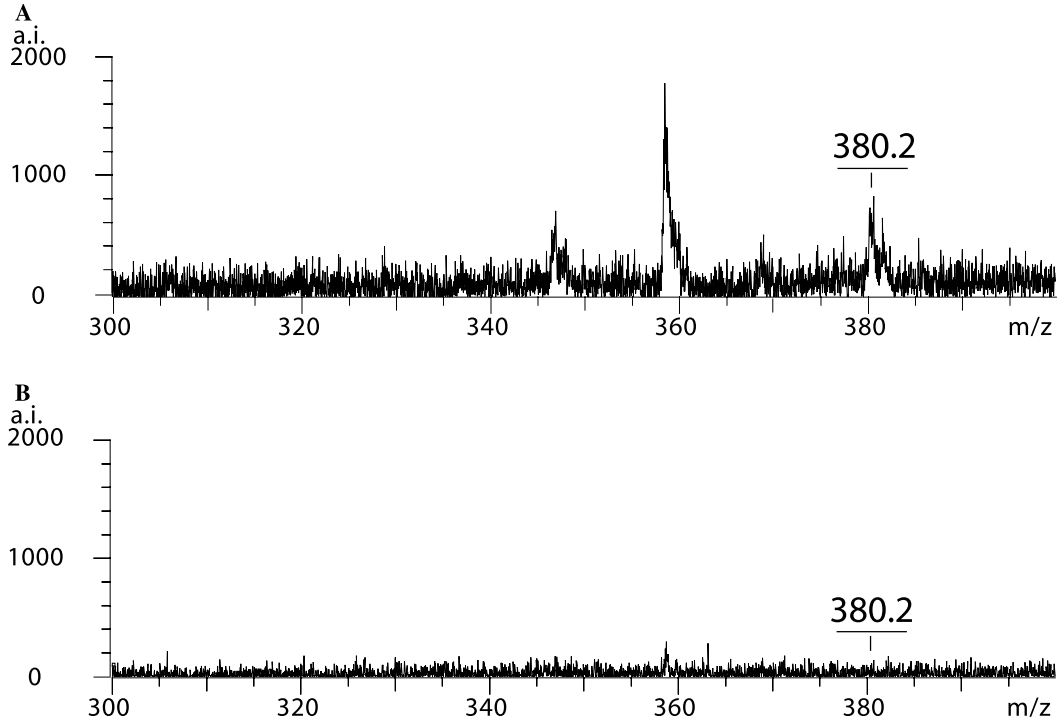

Fig. 6. The MALDI-PSD analysis of PBMCs incubated with AZT (A) revealed the expected signal of the unique fragment of AZT-TP at *m/z* 380.2. This signal was not detected in the MALDI-PSD analysis of PBMCs not incubated with AZT (B).

AZT-TP in standard solutions. Furthermore, we have to explore if MALDI-PSD analysis can be used for accurate quantification of AZT-TP by using an internal standard.

We conclude that: (1) AA/NA is a suitable matrix for MALDI-TOF analysis of NRTI-TPs, NTPs, and dNTPs. (2) AZT-TP, AT,P and dGTP can be detected up to 0.5 femtomole per sample by MALDI-TOF MS. (3) CTP, GTP, and UTP can be detected by MALDI-TOF MS. (4) Intracellular AZT-TP, ATP, and dGTP can be detected in PBMCs by MALDI-TOF MS. (5) Discrimination of AZT-TP from ATP and dGTP can be obtained by MALDI-PSD analysis in standard nucleotide solutions and in PBMCs. (6) Our developed method could be useful for NRTI-TP studies in HIV-1 infected children and adults.

Acknowledgment

We like to acknowledge the SKZ Foundation for financial support.

References

[1] A.S. Bergshoeff, P.L. Fraaij, A.M. van Rossum, T.F. Wolfs, S.P. Geelen, R. de Groot, D.M. Burger, Pharmacokinetics of nelfinavir in children: influencing factors and dose implications, Antiviral Ther. 8 (2003) 215–222.

[2] J.R. King, D.W. Kimberlin, G.M. Aldrovandi, et al., Antiretroviral pharmacokinetics in the paediatric population: a review, Clin. Pharmacokinet. 41 (2002) 1115–1133.

[3] D.M. Burger, A.M. van Rossum, P.W. Hugen, M.H. Suur, N.G. Hartwig, S.P. Geelen, H.J. Scherpbier, R.M. Hoetelmans, A.G. Vulto, R. de Groot, Dutch study group for children with HIV-1 infection, pharmacokinetics of the protease inhibitor indinavir in human immunodeficiency virus type 1-infected children, Antimicrob. Agents Chemother. 45 (2001) 701–705.

[4] A.M. van Rossum, R. de Groot, N.G. Hartwig, C.M. Weemaes, S. Head, D.M. Burger, Pharmacokinetics of indinavir and low-dose ritonavir in children with HIV-1 infection, AIDS 14 (2000) 2209–2210.

[5] P.L. Fraaij, A.S. Bergshoeff, A.M. van Rossum, N.G. Hartwig, D.M. Burger, R. de Groot, Changes in indinavir exposure over time: a case study in six HIV-1 infected children, J. Antimicrob. Chemother. 52 (2003) 727–730.

[6] D.M. Burger, R.M. Hoetelmans, P.W. Hugen, J.W. Mulder, P.L. Meenhorst, P.P. Koopmans, K. Brinkman, M. Keuter, W. Dolmans, Y.A. Hekster, Low plasma concentrations of indinavir are related to virological treatment failure in HIV-1-infected patients on indinavir-containing triple therapy, Antivir. Ther. 3 (1998) 215–220.

[7] C. Marzolini, A. Telenti, L.A. Decosterd, G. Greub, J. Biollaz, T. Buclin, Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients, AIDS 15 (2001) 71–75.

[8] A.I. Veldkamp, G.J. Weverling, J.M. Lange, J.S. Montaner, P. Reiss, D.A. Cooper, S. Vella, D. Hall, J.H. Beijnen, R.M. Hoetelmans, High exposure to nevirapine in plasma is associated with an improved virological response in HIV-1-infected individuals, AIDS 15 (2001) 1089–1095.

[9] M. Pfister, L. Labbe, S.M. Hammer, J. Mellors, K.K. Bennett, S. Rosenkranz, L.B. Sheiner, Adult AIDS clinical trial group study 398, population pharmacokinetics and pharmacodynamics of efavirenz, nelfinavir, and indinavir: adult AIDS clinical trial group study 398, Antimicrob. Agents Chemother. 47 (2003) 130–137.

[10] A.M. van Rossum, S.P. Geelen, N.G. Hartwig, T.F. Wolfs, C.M. Weemaes, H.J. Scherpbier, E.G. van Lochem, W.C. Hop, M. Schutten, A.D. Osterhaus, D.M. Burger, R. de Groot, Results of 2 years of treatment with protease-inhibitor-containing antiretroviral therapy in dutch children infected with human immunodeficiency virus type 1, Clin. Infect. Dis. 34 (2002) 1008–1016.

[11] M. Sale, L.B. Sheiner, P. Volberding, T.F. Blaschke, et al., Zidovudine response relationships in early human immunodeficiency virus infection, Clin. Pharmacol. Ther. 54 (1993) 556–566.

[12] W.Y. Gao, T. Shirasaka, D.G. Johns, et al., Differential phosphorylation of azidothymidine dideoxycytidine and dideoxyinosine in resting and activated peripheral blood mononuclear cells, J. Clin. Invest. 91 (1993) 2326–2333.

[13] C.F. Perno, R. Yarchoan, D.A. Cooney, N.R. Hartman, S. Gartner, M. Popovic, Z. Hao, T.L. Gerrard, Y.A. Wilson, D.G. Johns, Inhibition of human immunodeficiency virus (HIV-1/HTLV-IIIBa-L) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2′,3′-dideoxynucleosides, J. Exp. Med. 168 (1988) 1111–1125.

[14] E.S. Arner, S. Eriksson, Deoxycytidine and 2′,3′-dideoxycytidine metabolism in human monocyte- derived macrophages. A study of both anabolic and catabolic pathways, Biochem. Biophys. Res. Commun. 197 (1993) 1499–1504.

[15] C.V. Fletcher, E.P. Acosta, K. Henry, L.M. Page, C.R. Gross, S.P. Kawle, R.P. Remmel, A. Erice, H.H. Balfour Jr., Concentration-controlled zidovudine therapy, Clin. Pharmacol. Ther. 64 (1998) 331–338.

[16] H. Kuster, M. Vogt, B. Joos, V. Nadai, R. Luthy, A method for the quantification of intracellular zidovudine nucleotides, J. Infect. Dis. 164 (1991) 773–776.

[17] R.L. Robbins, B.H. Waibel, A. Fridland, Quantitation of intracellular zidovudine phosphates by use of combined cartridge-radioimmunoassay methodology, Antimicrob. Agents Chemother. 40 (1996) 2651–2654.

[18] S. Kewn, P.G. Hoggard, S.D. Sales, K. Jones, B. Maher, S.H. Khoo, D.J. Back, Development of enzymatic assays for quantification of intracellular lamivudine and carbovir triphosphate levels in peripheral blood mononuclear cells from human immunodeficiency virus-infected patients, Antimicrob. Agents Chemother. 46 (2002) 135–143.

[19] F. Becher, D. Schlemmer, A. Pruvost, M.C. Nevers, C. Goujard, S. Jorajuria, C. Guerreiro, T. Brossette, L. Lebeau, C. Creminon, J. Grassi, H. Benech, Development of a direct assay for measuring intracellular AZT triphosphate in humans peripheral blood mononuclear cells, Anal. Chem. 74 (2002) 4220–4227.

[20] J.D. Moore, G. Valette, A. Darque, X.J. Zhou, J.P. Sommadossi, Simultaneous quantitation of the 5′-triphosphate metabolites of zidovudine, lamivudine, and stavudine in peripheral mononuclear blood cells of HIV infected patients by high-performance liquid chromatography tandem mass spectrometry, J. Am. Soc. Mass Spectrom. 11 (2000) 1134–1143.

[21] E. Font, O. Rosario, J. Santana, H. Garcia, J.P. Sommadossi, J.F. Rodriguez, Determination of zidovudine triphosphate intracellular concentrations in peripheral blood mononuclear cells from human immunodeficiency virus- infected individuals by tandem mass spectrometry, Antimicrob. Agents Chemother. 43 (1999) 2964–2968.

[22] F. Becher, A. Pruvost, J. Gale, P. Couerbe, C. Goujard, V. Boutet, E. Ezan, J. Grassi, H. Benech, A strategy for liquid chromatography/tandem mass spectrometric assays of intracellular drugs:

application to the validation of the triphosphorylated anabolite of antiretrovirals in peripheral blood mononuclear cells, J. Mass Spectrom. 38 (2003) 879–890.

[23] D.A. Rideout, Bustamante, G. Siuzdak, Cationic drug analysis using matrix-assisted laser desorption/ionization mass spectrometry: application to influx kinetics, multidrug resistance, and intracellular chemical change, Proc. Natl. Acad. Sci. USA 90 (1993) 10226–10229.

[24] D.J. Harvey, Quantitative aspects of the matrix-assisted laser desorption mass spectrometry of complex oligosaccharides, Rapid Commun. Mass Spectrom. 7 (1993) 614–619.

[25] Y.C. Ling, L. Lin, Y.T. Chen, Quantitative analysis of antibiotics by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 12 (1998) 317–327.

[26] G. Siuzdak, Mass Spectrometry for Biotechnology, Academic Press, New York, 1996.

[27] J. Leushner, N.H. Chiu, Automated mass spectrometry: a revolutionary technology for clinical diagnostics, Mol. Diagn. 5 (2000) 341–348.

[28] D.A. van Ausdall, W.S. Marshall, Automated high-throughput mass spectrometric analysis of synthetic oligonucleotides, Anal. Biochem. 256 (1998) 220–228.

[29] A. Fridland, M.C. Connelly, R. Ashmun, Relationship of deoxynucleotide changes to inhibition of DNA synthesis induced by the antiretroviral agent 3′-azido-3′-deoxythymidine and release of its monophosphate by human lymphoid cells (CCRF-CEM), Mol. Pharmacol. 37 (1990) 665–670.