Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 848 (2007) 335–343
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous quantitation of the nucleotide analog adefovir, its phosphorylated anabolites and 2′-deoxyadenosine triphosphate by ion-pairing LC/MS/MS☆

Jennifer E. Vela, Loren Y. Olson, Alan Huang, Arnold Fridland, Adrian S. Ray*

Gilead Sciences, Foster City, CA 94404, USA

Received 26 June 2006; accepted 28 October 2006
Available online 13 November 2006

Abstract

The nucleotide analog adefovir is an important therapy for hepatitis B viral infection. The study of nucleoside/tide pharmacology has been hampered by difficulties encountered when trying to develop LC/MS/MS methods for these polar analytes. In an attempt to identify a more convenient, selective and sensitive alternative to the analysis of the metabolism of radiolabled parent nucleotide traditionally used for *in vitro* cell culture studies, an LC/MS/MS method was developed for the quantitative detection of adefovir and its phosphorylated metabolites in cellular samples. Ion-pairing reversed phase LC using tetrabutylammonium (TBA) and ammonium phosphate had the best compromise between chromatographic separation and positive mode MS/MS detection. Using microbore reverse phase columns and a low flow acetonitrile gradient it was possible to quantitate adefovir, its metabolites and 2′-deoxyadenosine triphosphate. A cross-validation showed comparable levels of adefovir and its metabolites were determined using either LC/MS/MS or radioactivity detection. However, initial methods were conducted at high pH and utilized an acetonitrile step gradient causing unacceptable column life and unpredictable equilibration. Further method optimization lowered the concentration of TBA and phosphate, decreased pH and applied a linear gradient of acetonitrile. This work resulted in a method that was found to have sensitivity, accuracy and precision sufficient to be a useful tool in the study of the intracellular pharmacology of adefovir *in vitro* and may be more broadly applicable.

Keywords: Nucleotide; LC/MS/MS; Ion pairing; Adefovir; Hepatitis B virus; Metabolism

1. Introduction

9-(2-Phosphonomethoxyethyl)adenine (adefovir), an acyclic nucleotide analogue of 2′-deoxyadenosine monophosphate, has potent antiviral activity against the hepatitis B virus (HBV). The addition of two pivalic acid moieties improves oral bioavailability [1,2], and in prodrug form adefovir dipivoxil (Hepsera®, Gilead Sciences, Inc.) is an efficacious therapy for HBV infection [3]. After cleavage by esterase mediated hydrolysis, adefovir enters cells and is anabolized to its active form, adefovir diphosphate (adefovir-DP) by kinases [4,5]. Adefovir-DP competes with 2′-deoxyadenosine triphosphate (dATP) for incorporation by the viral polymerase and after incorporation causes chain-termination of viral transcripts. In order to further the understanding of the intracellular metabolism of adefovir and other nucleoside and nucleotide analogs reliable quantitation methods are needed.

Mass spectrometry provides a detection method with sensitivity and specificity suitable for intracellular measurements but is not typically compatible with LC methods capable of resolving nucleotides. Nucleotides are difficult to analyze by traditional LC/MS/MS methodologies because their polar nature makes it difficult to obtain retention by reversed phased chromatography using typical aqueous–organic mobile phases. The use of anion exchange chromatography yields excellent peak shape and predictable retention times for nucleotides. However,

☆ Data reported in part as poster number ThPK 201 at the 52nd American Society for Mass Spectrometry (ASMS) Conference on Mass Spectrometry and Allied Topics held May 23–27, 2004 in Nashville Tennessee, USA.

* Corresponding author at: Department of Drug Metabolism, Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, CA 94404, USA. Tel.: +1 650 522 5536; fax: +1 650 522 1892.
E-mail address: adrian.ray@gilead.com (A.S. Ray).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.10.063

the high ionic strength normally needed to elute nucleotides from anion exchange columns requires salt concentrations that are not compatible with MS detection. There is one example of the use of weak anion exchange (WAX) liquid chromatography coupled to triple quadrupole MS/MS detection in the literature [6]. This method used a pH gradient to avoid the presence of high levels of salt for anion exchange chromatography. Porous graphitic carbon columns have also been shown to retain polar analytes including nucleotides, although retention mechanisms are more complex and retention not as predictable [7].

One way around the difficulties of detecting nucleotides is to dephosphorylate them to the nucleoside level before detection. Methods including dephosphorylation have been used successfully to quantitate nucleotide levels in patient samples. Common to these methods is the use of an initial chromatographic step to separate different phosphorylated forms from one another. Following fractionation the nucleotides can then be dephosphorylated using phosphatase and analyzed by radioimmunoassay (RIA) [8–14] ultra violet absorbance (UV) [15,16] or MS detection [17,18]. RIA offers sensitivity that rivals that of MS detection for many analytes. However, RIA requires the use of antibodies for each nucleotide being studied. In a few instances UV detection has been used for quanitation of nucleotides [15,16], but these methods have only been used for nucleoside analogs that show exceptionally efficient intracellular phosphorylation. The reason UV detection is not broadly applicable is the similar absorbance spectrum of abundant endogenous nucleotides and poor sensitivity. All of these methods include a time consuming sample preparation protocol with many steps before actual detection. Lengthy sample preparation limits throughput and raises concerns about nucleotide stability. In summary, methods using dephosphorylation suffer from complicated and inconvenient sample processing, low throughput, and, in the case of RIA, the necessity of developing specific antibodies.

The weaknesses of aforementioned methods for nucleotide detection have prompted the development of direct detection methods for nucleotides using LC/MS/MS. Ion-pairing reversed phase chromatography has been the most commonly used technique to facilitate chromatographic separation. Dimethyl-hexyl amine [19–25] or tetrabutylammonium (TBA) [26,27] have been used as ion-pairing reagents. Tetra-alkyl ammonium salts have usually been precluded from use in conjunction with MS detection because of problems with contamination of the analyzer, ion suppression and background interference. However, by limiting ion pair concentration and maintaining low flow rates, various methods using TBA have been shown to be well tolerated by modern ESI sources [26,27]. Surprisingly, similar lower limits of quantitation have been obtained from both negative [20–26] and positive mode ionization [6,19,27] MS/MS detection of the negatively charged nucleotide analogs. Results have shown that negative ion mode offers slightly greater sensitivity (two- to four-fold), while positive ion mode often generates unique fragmentation patterns, including the heterocyclic base of the nucleotide analog, resulting in lower potential for interference [6]. These methods have shown excellent retention, separation and sensitivity for nucleoside triphosphate analogs, but only limited attention has been given to the simultaneous detection of other phosphorylated intermediates of nucleosides.

While initial studies with some of the above mentioned methods showed promise for the detection of nucleoside triphosphate analogs, none were found to detect the parent and phosphorylated metabolites with sufficient sensitivity except ion pairing. In this report, a sensitive method for the detection of adefovir and its mono- and diphosphorylated anabolites, as well as its competing endogenous nucleotide dATP, in a single chromatographic separation using an ion-pairing LC/MS/MS method is presented. The method was developed to facilitate the analysis of phosphorylated metabolites generated during *in vitro* cell culture incubations with hepatic cells as a more convenient, sensitive and selective alternative to the traditional technique of using radiolabeled parent nucleotide. We have previously reported findings obtained using earlier versions of this method including the characterization of the intracellular hepatic metabolism of adefovir [28], a comparison of adefovir metabolism to a structurally related nucleotide analog [29] and the results obtained using adapted methods to study other nucleoside and nucleotide analogs [29–31]. Here observations made during method development, significant refinement to the previously reported method and the results of cross-validations are described.

2. Experimental

2.1. Reagents

2.1.1. Nucleotides

Adefovir (9-(2-phosphonomethoxyethyl)adenine) in its parent and diphosphate (DP) form and tenofovir-DP (9-(2-phosphonomethoxypropyl)adenine diphosphate) were synthesized by Gilead Sciences, Inc. (Foster City, CA, USA). Adefovir monophosphate (adefovir-MP) was synthesized as described previously [28]. The sodium salt of 2′-deoxyadenosine triphosphate (dATP) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and stable isotope labeled dATP (^{13}C, ^{15}N) was purchased from Spectra Stable Isotopes (Columbia, MD, USA). Radiolabeled adefovir ([adenine-2,8-^{3}H]-adefovir and [adenine-8-^{14}C]-adefovir) was obtained from Moravek Biochemical (Brea, CA, USA). Tritiated adefovir was ordered double purified by the vendor because of problems with contamination of the labeled material [28]. Concentration of nucleotide solutions were verified by their ultraviolet absorbance at 260 nm using the extinction coefficient for dATP ($15.3 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 7.0). Purity of nucleotides was verified by strong anion exchange chromatography on a mono Q HR 5/5 column. Stock solutions of 100 μM in deionized water were made for further dilution for working standards and quality control samples for LC/MS/MS analyses and stored at −20 °C when not in use.

2.1.2. Cell culture

Hep G2 cells were obtained from the American Tissue Type Culture Collection (number HB-8065) and maintained in Earle's

modified essential medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine, nonessential amino acids and penicillin-streptomycin (Irvine Scientific, Santa Ana, CA, USA). Cells were cultured in tissue culture treated T75 flasks and 12-well plates (BD Biosciences, Bedford, MA, USA) passaging twice a week using 2× trypsin treatment to dislodge cells.

2.1.3. Liquid chromatography

TBA hydroxide (TBAH) or TBA Acetate (TBAA, both obtained from Sigma–Aldrich, St. Louis, MO, USA) and monobasic ammonium phosphate were used to separate analytes on an Xterra MS, C18, 3.5 μM, 1.0 mm × 100 mm column (Waters Corporation, Milford, MA, USA), a YMC J'sphere, C18, 4 μm, 1.0 mm × 150 mm column (Waters Corporation) or a phenomenex Luna, C18(2), 3 μM, 1.0 mm × 100 mm column (Phenomenex, Torrance, CA, USA) during LC/MS/MS detection. Buffers for LC/MS/MS were filtered prior to the addition of acetonitrile through an Altech (Deerfield, IL, USA) vacuum filter flask apparatus using a nylon 0.2 μm filter. TBA bromide and dibasic potassium phosphate (Sigma–Aldrich) were used to separate radioactively labeled analytes on a Phenomenex Prodigy 5u ODS C18 column. All other chemicals were the highest grade available from Sigma–Aldrich.

2.2. Cell culture

Twenty-four hour incubations of 10 μM adefovir with Hep G2 cells were done essentially as previously described [28]. Briefly, either non-radiolabeled, ^{3}H (diluted with non-labeled adefovir to a specific activity of 1000 dpm/pmol) or ^{14}C (at a specific activity of approximately 100 dpm/pmol) adefovir were incubated with confluent monolayers of Hep G2 cells in 12-well tissue culture plates. After 24 h monolayers were washed twice with 5 ml of ice cold phosphate buffered saline. Each well of the cell culture plate was then scraped into 0.5 ml of 70% methanol and stored at −20 °C to facilitate extraction of intracellular metabolites. Methanol used for extracting samples for LC/MS/MS analyses contained 2 pmol tenofovir-DP per 1×10^5 Hep G2 cells, as an internal standard.

2.3. Sample preparation

Cellular debris from samples were removed by spinning at 15,000 × *g* in a microcentrifuge and transferring the supernatant to a fresh tube. Samples were then dried in a speed vacuum system. Dried samples were then resuspended in either 80 mM TBAH with 4 mM ammonium phosphate (for initial LC/MS/MS analysis), 20 mM TBAA (for optimized LC/MS/MS analysis) or water (for radioactivity analysis) at a concentration of 1×10^5 cells/10 μl. A 0.45 μm Gelman Acrodisc LC PVDF syringe filter (PAL corporation, East Hill, NY, USA) was used to filter the injection buffers to remove any particulate matter. Sample stability in the LC/MS/MS injection buffer was found to be greater than 24 h at 4 °C based on multiple injections of the same sample from a cooled autosampler.

2.4. Instrumentation

Samples were injected by a CTC Analytics LEAP autosampler (Leap Technologies, Carrboro, NC, USA). A Shimadzu LC-20AD (Shimadzu Scientific Instruments, Columbia, MD, USA) tertiary high performance liquid chromatography system with a 5 μl ASI static mixer (Analytical Scientific Instruments, El Sobrante, CA, USA) was used to maintain 40–50 μl/min flow rates and mediate the gradient. Peek tubing (Upchurch Scientific) with 0.062 μm × 125 μm (0.005 in.) internal diameter was used throughout allowing for a dead volume of approximately 100 μl (T_0 of 2 min). A 10 port Valco valve (Valco Instruments Co., Inc., Houston, TX, USA) was used to direct flow to either the mass spectrometer or to waste. The third pump was attached separately to the valve giving a constant flow of 200 μl/min 20% acetonitrile to the mass spectrometer source when the LC flow was diverted to waste in order to facilitate cleaning. The LC system was connected to an API 4000 triple quadrupole mass spectrometer running in positive ion multiple reaction monitoring (MRM) mode (Applied Biosystems/MDS Sciex, Foster City, CA, USA).

2.5. Liquid chromatography conditions

2.5.1. Ion-pairing LC conditions for MS/MS detection

We have previously described the modification of an earlier reported iosocratic ion-pairing reversed phase chromatographic method used for the detection of nucleoside triphosphates [27] to include an acetonitrile step gradient to allow for the analysis of adefovir, adefovir-MP, adefovir-DP and tenofovir-DP (internal standard) in a single chromatographic separation [28]. After preparation, samples of 1×10^5 cells resuspended in 10 μl of either 80 mM TBAH and 4 mM ammonium phosphate (for initial methods using an Xterra column [28]) or 20 mM TBAA (for optimized methods using either a YMC or Luna column) were injected on column. The initial method used an Xterra column and a flow rate of 50 μl/min with a step gradient from mobile phase A containing 0.25 mM TBAH, 4 mM ammonium phosphate (pH 6.0) and 6% acetonitrile (isocratic for 5 min) to mobile phase B containing 0.25 mM TBAH, 4 mM ammonium phosphate (pH 6.0) and 20% acetonitrile for 23 min followed by 7 min re-equilibration to 100% mobile phase A (for a total analysis time of 30 min) [28]. Further optimization to lower pH reported here allowed for the use of a YMC or Luna column and a flow rate of 40 μl/min with a linear gradient from 100% mobile phase A containing 0.2 mM TBAA, 2 mM ammonium phosphate (pH 6.0) and 5% acetonitrile (held isocratic for the first 2 min) to 80% mobile phase B containing 50% acetonitrile and 2 mM ammonium phosphate (pH 4.0) over 25 min followed by 5 min re-equilibration to 100% mobile phase A. Flow was direct from the column to the mass spectrometer by activating the switching valve 2 min prior to analyte elution and directed back to waste 2 min post elution to allow for slight changes in retention. Syringe wash solutions were 1% formic acid in water and 1% formic acid in 50% acetonitrile. The syringe was washed with the aqueous solution before each injection. After injections the syringe and injection port were washed once with the aqueous

Table 1
Summary of general MS parameters

Probe height	8 mm
Curtain gas (CUR)	10 psi
Collision gas (CAD)	4.0 psi
Ion spray voltage (IS)	5500 V
Temperature	200 °C
Polarity	Positive
Ion source gas 1 (GS1)	10.0 psi
Ion source gas 2 (GS2)	30.0 psi
Resolution Q1	Low
Resolution Q3	Low
MR pause between scans	5 ms
Dwell time	500 ms
Entrance potential	10 V
Declustering potential (DP)	196 V[a]

[a] Declustering potential for adefovir, adefovir-MP and adefovir-DP, optimal values for dATP and stable isotope dATP found to be 146 V.

and acetonitrile wash solutions. Carry over observed between analytical separations was less than 1.5% for all analytes. To allow for optimal lower limits of quantitation two blank injects were used following high standards and quality control samples. Cell culture sample sets were also run in reverse order allowing for the accurate quantitation of samples with lower analyte levels prior to those with higher levels.

2.5.2. Radiochromatography

Studies using radiolabeled adefovir for detection were analyzed using reversed phase ion-pairing chromatography on a Phenomenex Prodigy column, fraction collection and scintillation counting as previously described [28].

2.6. Mass spectrometer conditions

2.6.1. MS/MS conditions

The API 4000 mass spectrometer in positive MRM mode was set to the general parameters listed in Table 1 and analyte specific parameters listed in Table 2.

2.6.2. Quantitation methods

For quantitation 7 point standard curves were made in blank matrices covering 3 orders of magnitude of analyte concentration. In all cases linearity exceeded an r^2 value of 0.99. To assure precision within 20% over the course of the analysis, standard curve samples were injected at the beginning and end of the sample set.

3. Results and discussion

A sample extraction including lyses of cells at −20 °C in 70% methanol was chosen based on extensive use of this method in the literature. We have tried alternative methods using acetonitrile in the presence or absence of acetic acid but have always observed similar or lower endogenous nucleotide extraction. When developing an extraction method care should be taken to avoid high organic levels as this will cause the nucleotides to crash out of solution. The effectiveness of this procedure in cellular lyses and extraction was evident in the measurement of intracellular dATP levels consistent with those reported in numerous reports using different extraction and detection techniques (see discussion below). Near complete recovery of adefovir and its phosphorylated metabolites from samples by the extraction procedure was inferred based on the similarity in detected amounts between cell samples spiked prior to extraction with analytes and samples generated by adding analytes directly into the injection buffer. A formal freeze thaw validation was not done but degradation has not been evident in samples stored at −20 °C in 70% methanol, as dried pellets, or in TBA injection buffer.

The mass spectrometer was tuned for adefovir, adefovir-MP, adefovir-DP, tenofovir-DP, dATP and stable isotope dATP by 10 μl/min infusions of 200–1000 nM analyte resuspended in 20% acetonitrile and 0.1 mM TBAA. Product ion scans in positive ionization mode showed that a unique product ion was formed as the major fragment for adefovir, adefovir-MP, adefovir-DP and tenofovir-DP (fragmentation patterns shown in Fig. 1). The generation of unique fragments is an advantage of positive ionization LC/MS/MS detection of nucleotides and is one of the reasons we chose to pursue a positive ion method (similar observation made for another nucleotide analog by others [6]). During tuning it was found that enhanced signal was obtained from 20% acetonitrile in the presence of 0.1 mM TBAA. Common parameters for all analytes are listed in Table 1 and analyte specific parameters are presented in Table 2. Based on prioritizing the accurate quantitation of the active species adefovir-DP and its competing natural nucleotide dATP, tenofovir-DP was chosen as an internal standard due to its similar ionization and retention characteristics. The choice of internal standard likely resulted in sacrificed performance for adefovir and adefovir-MP. Nevertheless, we observed acceptable % theoretical values and reproducibility for the intended purpose of the assay (generally within 35% and 25% for independently prepared quality controls and standards at or near the lower limit of quantitation and at higher concentrations, respectively). Inclu-

Table 2
Summary of analyte specific MS parameters

Analyte	Parameter		
	Mass transition [*m*/*z*]	Collision energy (CE) [V]	Collision cell exit potential (CXP) [V]
Adefovir	274.2/162.4	38	12
Adefovir-MP	354.0/162.4	51	16
Adefovir-DP	434.0/162.4	65	12
Tenofovir-DP	448.3/176.4	51	12
dATP (stable isotope)	492.1(506.7)/136.2(145.6)	27	10

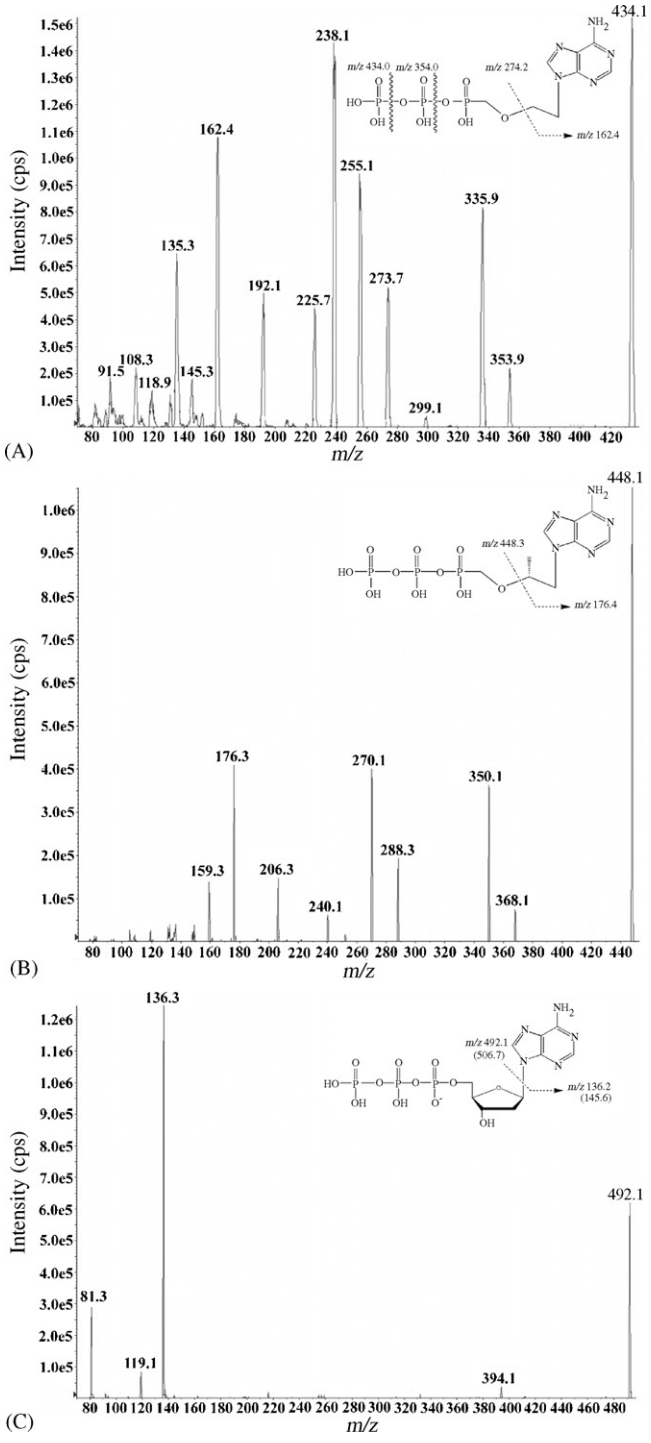

Fig. 1. Product ion spectra obtained from 10 μl/min infusions of 1 μM analyte in 20% acetonitrile and 0.1 mM TBAA monitoring with positive ionization mode using multiple cycle averaging (MCA) while ramping collision energy. (A) Product ion spectra of adefovir-DP including fragmentation to adefovir (observed at *m/z* 273.7) and adefovir-MP (observed at *m/z* 353.9). Following optimization the most abundant fragment observed for adefovir (optimized *m/z* 274.2), adefovir-MP (optimized *m/z* 354.0) and adefovir-DP (optimized *m/z* 434.0) was *m/z* 162.4 (fragmentation pattern depicted in inset). (B) Product ion spectra of tenofovir-DP and the site of fragmentation used for MS/MS detection. (C) Product ion spectra of dATP and its fragmentation to adenine. In parenthesis is the *m/z* transition used for stable isotope labeled (C^{13}, N^{15}) dATP.

sion of internal standards with similar retention to adefovir and adefovir-MP would likely further improve performance for these analytes.

Chromatography times of 30 min and the extensive equilibration needed for ion-pairing makes LC optimization challenging. In the mobile phase it was found that increased concentrations of ammonium phosphate (optimized between 0 and 6 mM) and decreased pH served to increase elution of phosphorylated metabolites and improve peak shape. Optimization of the mobile phase pH was done between 3.7 and 12. Surprisingly, lower pH did not improve the positive mode LC/MS/MS signal perhaps giving some clue as to the mode of ionization responsible for the signal obtained from nucleotides in positive ionization mode. Decreasing pH below 6.0 caused adefovir to have insufficient retention time and its signal was severely suppressed by contents of the injection (ion suppression discussed later).

Initial studies showed that ion pair concentrations in the injection buffer as low as 5 mM TBA could be used for neat samples, however, in the presence of cellular matrices, higher amounts of ion pair were required to minimize peak broadening and splitting for adefovir and its metabolites. Optimization illustrated that ion-pairing interactions are established in the inject buffer and low levels of ion pair in the mobile phase are only needed to maintain column equilibration. As reported previously, under similar conditions the column life for the Xterra column is relatively short [27], allowing for optimal peak shape for the first 100 injections followed by continuous decline in chromatographic performance. The use of different columns was precluded because the 80 mM TBAH containing injection buffer is approximately pH 10 and the Xterra is one of the few columns with claimed stability under highly alkaline conditions. Therefore, it was a goal to decrease the pH to allow for different capillary columns to be used. Further optimization showed that 20 mM TBAA could be used in the inject buffer with minimal deleterious effects on peak shape. This inject buffer was found to have a pH close to neutral and allowed for the use of different columns. Various C18 columns including the Xterra, YMC and Luna were tested with the new mobile phase and injection buffers but the best peak shape and column life was observed for the Luna. Based on experience with numerous Luna columns under our final conditions, noticeable changes in peak shape have not been observed until in-excess of 500 injections.

The obtained method allowed for detection of adefovir and its phosphorylated metabolites in cellular matrices (Fig. 2A). Comparison of neat and matrix containing injections showed only a mild matrix effect, consisting of slight peak broadening, with no loss in MS response. The lack of a matrix effect on MS signal probably contributes to the precision of this method (discussed below) and may be an advantage over anion exchange methods which often suffer from limited capacity and matrix dependent ion suppression [6]. Linearity of standard curves covering in excess of 3-orders of magnitude was consistently above an r^2 value of 0.99 and standards run at the end of the run did not vary by more than 15% from the initial standard curve. Lower limits of quantitation, defined as allowing for acceptable accuracy and precision and generally having peak heights five-fold greater than background, were observed to be between 25 and

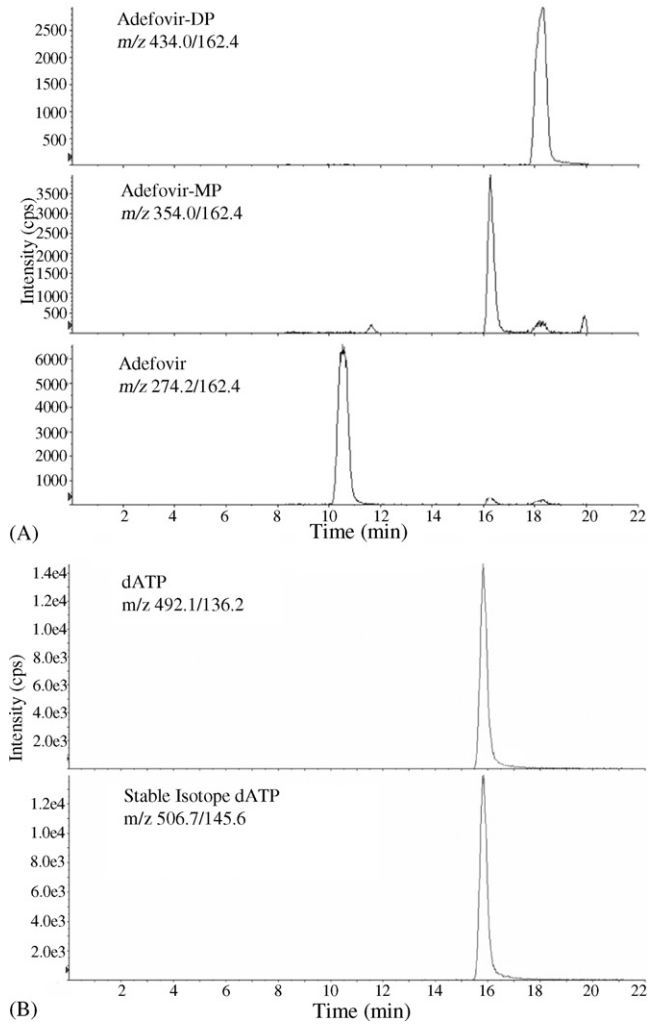

Fig. 2. Separation and detection of analytes using TBAA ion pair and a linear gradient of acetonitrile on a YMC column coupled to positive mode MS/MS. (A) Chromatographic separation and detection of adefovir (bottom panel), adefovir-MP (middle panel) and adefovir-DP (top panel) in cellular matrices from Hep G2 cells. Samples were generated by adding 200 nM of adefovir and its phosphorylated metabolites to matrices from 1×10^5 cells/10 μl TBAA injection buffer and injecting 10 μl on column. Source fragmentation from adefovir-MP to adefovir and from adefovir-DP to adefovir-MP and adefovir is apparent in the respective MRMs depicted. (B) Chromatographic and MS signal similarity between dATP (top panel) and stable isotope labled dATP (bottom panel). Sample was generated by adding 200 nM dATP and stable isotope labeled dATP to TBAA injection buffer and injecting 10 μl on column.

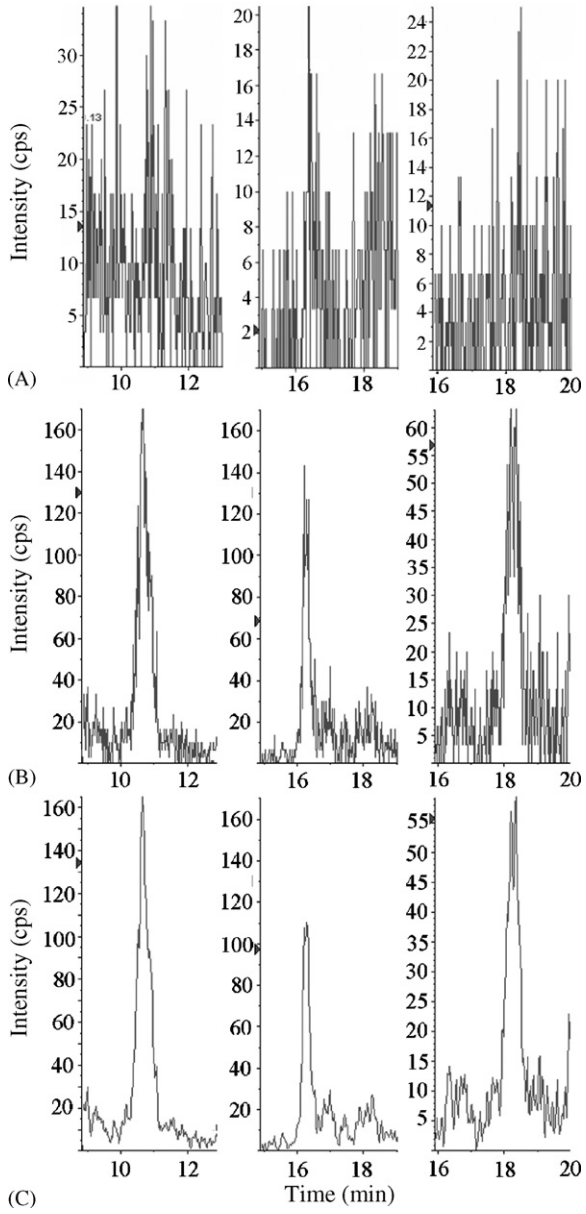

Fig. 3. Assessment of selectivity and lower limits of quantitation. An injection of extract from 1×10^5 Hep G2 cells either in the absence (panel A) or presence of added adefovir, adefovir-MP and adefovir-DP (panels B and C) at or near their lower limits of quantitation (25, 50 and 25 fmols on column, respectively) on an Xterra column. Both the unsmoothed chromatograms (panel B) and chromatograms with a gaussian smooth of 10 applied (panel C) are presented for samples spiked with adefovir and its phosphorylated metaoblites.

50 fmols on column for adefovir and its anabolites (Fig. 3). Slightly more sensitivity was observed for natural and stable isotope labeled dATP of approximately 20 fmols on column (data not shown). These levels of sensitivity were sufficient to study the metabolism of 10 μM adefovir in hepatic cells [28,29] and are also anticipated to be capable of detecting expected levels of many nucleotide analogs *in vivo* (often present at low μM intracellular levels).

The method was cross-validated for use in measuring *in vitro* samples by two different sets of experiments. Triplicate injections of the same sample of Hep G2 extract from cells treated for 24 h with adefovir resulted in coefficient of variation (CV) not greater than 10% for each of the analytes. Comparison of levels from the three independent samples resulted in CV values of 3.68, 21.3 and 9.88% for adefovir, adefovir-MP and adefovir-DP, respectively. The precision observed upon injection of the independently generated samples is consistent with variation often seen in cell culture assays and probably arises from error introduced by differences in cell growth, metabolism and sample preparation, with only a minor contribution from the analytical method. The second cross-validation compares the more traditional method of studying *in vitro* cellular metabolism using radiolabeled nucleotides for quantitation with LC/MS/MS techniques. Data summarized in Table 3 shows the comparison

Table 3
Comparison of results for adefovir metabolite concentrations in Hep G2 cells after a 24 h incubation at 10 μM adefovir as determined by radioactively labeled adefovir or LC/MS/MS

	pmols/million cells		
	^{3}H	^{14}C	MS/MS
Adefovir	10.8 ± 2.2	11.6 ± 1.1	14.3 ± 5.9
Adefovir-MP	3.00 ± 1.86	2.70 ± 0.43	2.50 ± 0.73
Adefovir-DP	7.35 ± 4.33	5.8 ± 0.55	6.19 ± 1.66

Values represent the mean ± S.D. of three independent experiments for ^{3}H and two independent experiments done in triplicate for ^{14}C and LC/MS/MS.

between the three different analyses. No marked difference was noted between quantitation methods (variation less than 25% with overlapping values when taking into account their standard deviations). These results illustrate that the LC/MS/MS method yielded comparable results to radiolabeled techniques, suggesting a high level of accuracy for both methods. The LC/MS/MS method offered the advantage of an added level of selectivity imparted by MS/MS detection. Selectivity is a common problem when using radiolabeled parent nucleosides and nucleotides because of instability in the radiolabel and difficulties in analytically separating the contaminating radiolabeled peaks. This can be especially problematic when measuring nucleotides because it is common for the radiolabel to enter the natural nucleoside and nucleotide pools causing high levels of radiolabeled endogenous nucleosides and nucleotides with similar chromatographic behavior to the analytes of interest [28,32,33].

After metabolism to its diphosphate form, adefovir competes with dATP for incorporation by the HBV viral polymerase. The intracellular levels of dATP are, therefore, an important factor in understanding the intracellular pharmacology of adefovir. Measuring the levels of an endogenous molecule poses some problems because it is hard to make standard curves in the presence of cellular matrices. We wanted to test the viability of a method using stable isotope labeled nucleotides in the quantitation of endogenous nucleotide pools. Stocks of dATP and stable isotope labeled dATP were carefully characterized for concentration and purity by 260 nm absorbance and LC/UV. MS parameters for dATP and stable isotope labeled dATP were identical with the exception of *m/z* ratios and similar to those observed for the acyclic phosphonate analogs studied (Tables 1 and 2). Lower optimized collision energy relative to acyclic nucleotides may indicate the instability of the glycosidic linkage being fragmented in dATP in its natural and stable isotope forms (Fig. 1C). LC/MS/MS analysis of a neat inject of dATP and stable isotope labeled dATP yielded identical signal and retention time during the low flow ion-pairing chromatographic separation (Fig. 2B).

To compare results obtained from stable isotope methods with other techniques, the amount of dATP in Hep G2 cells was quantitated by different methodologies. Quantitation of cellular dATP levels based on a neat dATP and stable isotope dATP standard curve resulted in a level of 13.1 ± 0.3 and 12.6 ± 0.3 pmols/million cells (mean ± S.D. of three independently generated samples), respectively. Standard curves from stable isotope labeled dATP in cellular matrices yielded levels of 12.6 ± 0.3 pmols/million cells. The reason for the similarity in the neat and matrix containing quantitation methods was that, similar to the acyclic nucleotide analogs studied, there was only a slight chromatographic matrix effect observed. As a final assessment of the method, a standard curve of dATP was added to cellular matrices (containing the normal level of endogenous dATP) and the *y*-intercept determined. The data was in good agreement with other analytical methods indicating a concentration of 12.2 pmols/million cells. In a review of intracellular nucleotide levels by Traut the level of dATP found in tumor cell lines is reported to be 23 ± 22 μM [34]. Estimating an intracellular volume of 1–2 pl/cell for a liver cell line (for example primary rat hepatocytes have been found to be 1 pl/cell [35]), this value is in reasonable agreement with our measurements (between 6 and 12 μM). These data suggest that in cellular matrices from Hep G2 cells that all of the methods give a reasonable assessment of dATP concentrations. In subsequent studies, we have chosen to use the stable isotope method because it gives the possibility of correcting for matrix effects that may arise from different preparation procedures and cellular matrices.

While the described ion-pairing LC/MS/MS method was able to accurately quantitate intracellular nucleotide levels, some limitations were observed due to the choice of mobile phase and injection buffer components. The required use of large amounts of ion pair in the injection buffer causes ion suppression. As shown in Fig. 4, areas of severe ion suppression during the initial part of the analysis are present. The presence of a second, less pronounced, area of ion suppression appearing after 11 min in the chromatogram was also observed. Differing levels of ion suppression may explain the differential sensitivity and precision observed for the analytes. Phosphate clusters and their ammoni-

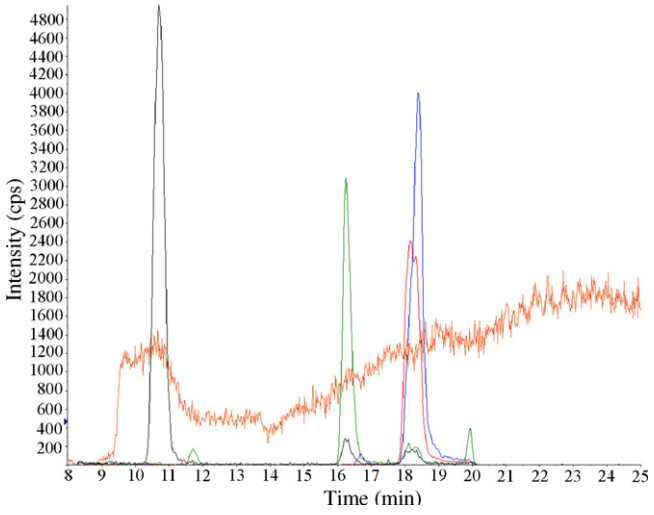

Fig. 4. Ion suppression during chromatography was observed by infusing 10 μl/min 1 μM solution of adefovir-DP post column during a normal ion-pairing LC gradient. The trace of adefovir-DP signal was then overlaid on the LC/MS/MS chromatogram of an injection of 200 nM (2 pmols on column) adefovir (black), adefovir-MP (green), adefovir-DP (red) and tenofovir-DP (blue) spiked into cellular matrices from 1×10^{5} Hep G2 cells and separated on an Xterra column to illustrate the position of ion suppression relative to the elution of analytes. Similar ion suppression profiles were observed during infusion of all analytes and under all column and gradient conditions studied.

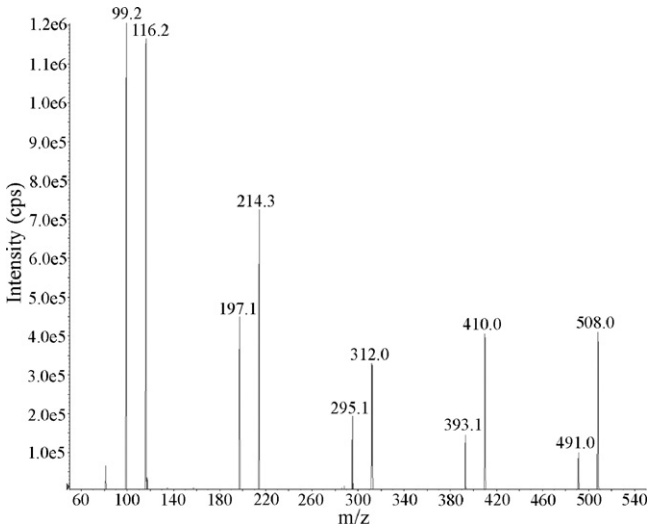

Fig. 5. Detection of phosphate clusters, a potential cause of interference during ion-pairing LC/MS/MS. Shown is the product ion spectra of an ammoniated adduct of $(H_3PO_4)_5$. Product ion spectra generated by infusing mobile phase A at 10 μl/min and selecting the products of the peak with a *m/z* of 508.0 and ramping collision energy in multiple cycle averaging (MCA) mode. Closely corresponding to the $(H_3PO_4)_5$ cluster and its ammoniated adduct, the *m/z* of dATP and ATP are 492.1 and 508.0, respectively.

ated adducts have also been observed creating a number of high intensity peaks that can cause interference for some analytes (Fig. 5).

4. Conclusions

We have presented an LC/MS/MS method illustrated by cross-validations to have sensitivity, precision and accuracy rivaling that of traditional radioactivity methods when applied to the measurement of the intracellular metabolism of adefovir to its phosphorylated anabolites *in vitro*. Significant advantages have also been observed in terms of selectivity, as LC/MS/MS detection alleviated interference peaks caused by impurities in radiolabled adefovir (interference peaks were previously reported from metabolite identification studies [28]). The earlier version of this method using a step gradient and TBAH mobile phases and injection buffer facilitated a complete study of the *in vitro* intracellular pharmacology of adefovir in hepatic cells [28] and a comparison of adefovir metabolism to that of a structurally related nucleotide analog [29]. The detailed results from the method development and cross-validations described in this manuscript should further the ability to study adefovir as well as aid in the development of methods for different analytes and matrices. The further development of analytical techniques for nucleotide detection should facilitate the further study of adefovir and other nucleoside and nucleotide analogs both *in vitro* and *in vivo*, contributing to a better understanding of their intracellular pharmacology.

Acknowledgements

We would like to thank Robert L. St. Claire III for guidance on ion-pairing LC/MS/MS, and Eugene Eisenberg, Xubin Zheng, Matthew Wright, Gerald Rhodes, and Mick Hitchcock for thoughtful review of the manuscript. Work supported by Gilead Sciences, Inc.

References

[1] L. Naesens, J. Balzarini, N. Bischofberger, E. De Clercq, Antimicrob. Agents Chemother. 40 (1996) 22.
[2] R.V. Srinivas, B.L. Robbins, M.C. Connelly, Y.F. Gong, N. Bischofberger, A. Fridland, Antimicrob. Agents Chemother. 37 (1993) 2247.
[3] T. Dando, G. Plosker, Drugs 63 (2003) 2215.
[4] R. Krejcova, K. Horska, I. Votruba, A. Holy, Collect. Czech. Chem. Commun. 65 (2000) 1653.
[5] B.L. Robbins, J. Greenhaw, M.C. Connelly, A. Fridland, Antimicrob. Agents Chemother. 39 (1995) 2304.
[6] G. Shi, J.T. Wu, Y. Li, R. Geleziunas, K. Gallagher, T. Emm, T. Olah, S. Unger, Rapid Commun. Mass Spectrom. 16 (2002) 1092.
[7] J. Xing, A. Apedo, A. Tymiak, N. Zhao, Rapid Commun. Mass Spectrom. 18 (2004) 1599.
[8] J.T. Slusher, S.K. Kuwahara, F.M. Hamzeh, L.D. Lewis, D.M. Kornhauser, P.S. Lietman, Antimicrob. Agents Chemother. 36 (1992) 2473.
[9] K. Peter, J.P. Lalezari, J.G. Gambertoglio, J. Pharm. Biomed. Anal. 14 (1996) 491.
[10] B.L. Robbins, J. Rodman, C. McDonald, R.V. Srinivas, P.M. Flynn, A. Fridland, Antimicrob. Agents Chemother. 38 (1994) 115.
[11] B.L. Robbins, B.H. Waibel, A. Fridland, Antimicrob. Agents Chemother. 40 (1996) 2651.
[12] B.L. Robbins, T.T. Tran, F.H. Pinkerton Jr., F. Akeb, R. Guedj, J. Grassi, D. Lancaster, A. Fridland, Antimicrob. Agents Chemother. 42 (1998) 2656.
[13] T.T. Tran, B.L. Robbins, F.H. Pinkerton Jr., B. Ferrua, J. Grassi, A. Fridland, Antiviral Res. 58 (2003) 125.
[14] K.H. Moore, J.E. Barrett, S. Shaw, G.E. Pakes, R. Churchus, A. Kapoor, J. Lloyd, M.G. Barry, D. Back, AIDS 13 (1999) 2239.
[15] C. Solas, Y.F. Li, M.Y. Xie, J.P. Sommadossi, X.J. Zhou, Antimicrob. Agents Chemother. 42 (1998) 2989.
[16] A. Darque, G. Valette, F. Rousseau, L.H. Wang, J.P. Sommadossi, X.J. Zhou, Antimicrob. Agents Chemother. 43 (1999) 2245.
[17] J.D. Moore, G. Valette, A. Darque, X.J. Zhou, J.P. Sommadossi, J. Am. Soc. Mass. Spectrom. 11 (2000) 1134.
[18] T. King, L. Bushman, J. Kiser, P.L. Anderson, M. Ray, T. Delahunty, C.V. Fletcher, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 843 (2006) 147.
[19] E.N. Fung, Z. Cai, T.C. Burnette, A.K. Sinhababu, J. Chromatogr. B Biomed. Sci. Appl. 754 (2001) 285.
[20] A. Pruvost, F. Becher, P. Bardouille, C. Guerrero, C. Creminon, J.F. Delfraissy, C. Goujard, J. Grassi, H. Benech, Rapid Commun. Mass Spectrom. 15 (2001) 1401.
[21] G. Hennere, F. Becher, A. Pruvost, C. Goujard, J. Grassi, H. Benech, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 789 (2003) 273.
[22] F. Becher, A. Pruvost, J. Gale, P. Couerbe, C. Goujard, V. Boutet, E. Ezan, J. Grassi, H. Benech, J. Mass Spectrom. 38 (2003) 879.
[23] F. Becher, A.G. Pruvost, D.D. Schlemmer, C.A. Creminon, C.M. Goujard, J.F. Delfraissy, H.C. Benech, J.J. Grassi, AIDS 17 (2003) 555.
[24] F. Becher, A. Pruvost, C. Goujard, C. Guerreiro, J.F. Delfraissy, J. Grassi, H. Benech, Rapid Commun. Mass Spectrom. 16 (2002) 555.
[25] 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, Anal. Chem. 74 (2002) 4220.
[26] E. Witters, W. Van Dongen, E.L. Esmans, H.A. Van Onckelen, J. Chromatogr. B Biomed. Sci. Appl. 694 (1997) 55.
[27] R.L. St Claire 3rd, Rapid Commun. Mass Spectrom. 14 (2000) 1625.
[28] A.S. Ray, J.E. Vela, L. Olson, A. Fridland, Biochem. Pharmacol. 68 (2004) 1825.
[29] W.E.t. Delaney, A.S. Ray, H. Yang, X. Qi, S. Xiong, Y. Zhu, M.D. Miller, Antimicrob. Agents Chemother. 50 (2006) 2471.
[30] A.S. Ray, F. Myrick, J.E. Vela, L. Olson, E.J. Eisenberg, K. Borroto-Esoda, M.D. Miller, A. Fridland, Antivir. Ther. 10 (2005) 451.

[31] K. Borroto-Esoda, J.E. Vela, F. Myrick, A.S. Ray, M.D. Miller, Antivir. Ther. 11 (2006) 377.
[32] G. Ahluwalia, D.A. Cooney, H. Mitsuya, A. Fridland, K.P. Flora, Z. Hao, M. Dalal, S. Broder, D.G. Johns, Biochem. Pharmacol. 36 (1987) 3797.
[33] A.S. Ray, L. Olson, A. Fridland, Antimicrob. Agents Chemother. 48 (2004) 1089.
[34] T.W. Traut, Mol. Cell Biochem. 140 (1994) 1.
[35] B. Andersen, A. Rassov, N. Westergaard, K. Lundgren, Biochem. J. 342 (Pt 3) (1999) 545.