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Simultaneous determination of Ziagen and its phosphorylated metabolites by ion-pairing high-performance liquid chromatography–tandem mass spectrometry

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Abstract

An ion-paring HPLC–MS–MS method with positive ion mode electrospray ionization has been developed to simultaneously quantify Ziagen, carbovir monophosphate, carbovir diphosphate and carbovir triphosphate. *N′*,*N′*-Dimethylhexylamine was used as the ion-pairing agent. The presence of this ion-pairing agent allowed the retention and separation of the four compounds on a reversed-phase HPLC column as well as the detection of the nucleotides with positive ion mode electrospray ionization. The limits of detection were found to be better than 25 n*M* for all the analytes. Calibration curves of the analytes showed excellent linearity over the range of 25 n*M* to 5 μ*M*. The relative standard deviations and accuracies for replicate analyses of quality control samples were less than 15%. The method has been successfully applied to the analysis of these compounds in human liver cells treated with Ziagen.

Keywords: Ziagen; Carbovir phosphate

1. Introduction

Ziagen (abacavir, 1592U89) (Fig. 1) is a carbocyclic nucleoside analog with in vitro activity and in vivo efficacy against the human immunodeficiency virus (HIV) [1–4]. Like other nucleoside analogs, its antiviral activity is dependent upon intracellular metabolism to a nucleotide, i.e., triphosphate, analog. The anabolic pathway from Ziagen to its active form, carbovir triphosphate (CBV-TP), involves three key intermediates: Ziagen monophosphate (Ziagen-MP), carbovir monophosphate (CBV-MP) and carbovir diphosphate (CBV-DP) [1,2]. Determining the intracellular levels of a nucleoside analog and its anabolites is crucial to understanding both its mechanisms of activation and its pharmacodynamics. However, accurate quantitation of nucleotide analogs is complicated by the presence of numerous endogenous nucleotides, such as adenosine 5′-triphosphate (ATP) and 2′-deoxyguanosine 5′-triphosphate (dGTP), due to their structural similarity (Fig. 1) and relatively high concentrations [5]. Their presence makes separation of the phosphorylated metabolites from the endogenous nucleotides a necessity for accurate and sensitive quantitation.

Strong anion-exchange high-performance liquid chromatography (HPLC) has been used to isolate the

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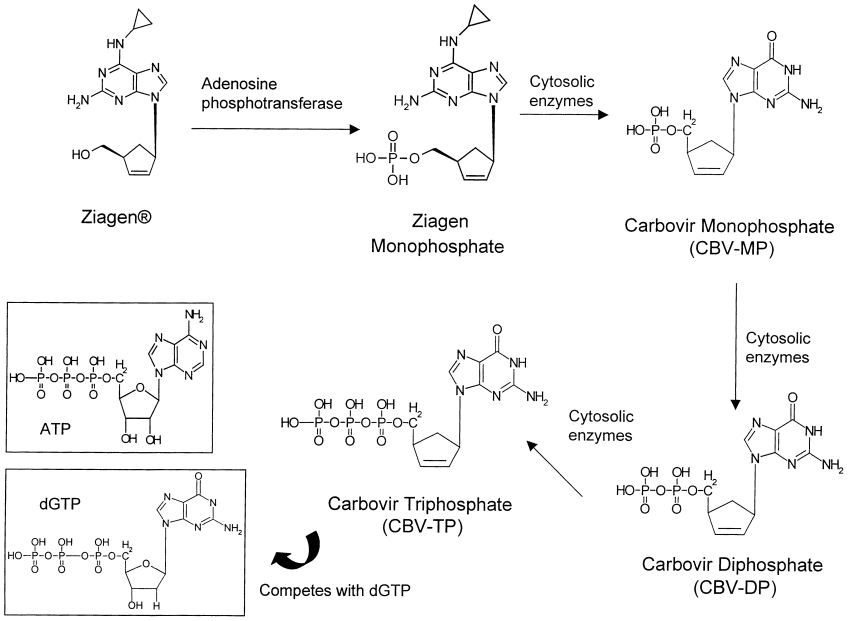

Fig. 1. Metabolic pathway for the anabolism of Ziagen to carbovir triphosphate (CBV-TP) via Ziagen monophosphate, carbovir diphosphate (CBV-DP) and carbovir monophosphate (CBV-MP). CBV-TP is the competitor of endogenous dGTP. ATP is present in high concentrations in cells. (Adapted from Ref. [2]).

nucleotides (for example, see Ref. [2]), but because of the long run times (≥1 h per sample) and requirement for radiometric detection, its utility has been limited mainly to in vitro studies. The power of HPLC coupled with mass spectrometry (MS), which has become widely used for high-throughput analyses in pharmaceutical industry [6–8], makes it an attractive alternative. However, nucleotides and oligonucleotides, having one or more charges, pose a unique challenge to conventional reversed-phase HPLC and HPLC–MS analyses: establishing chromatographic conditions which provide adequate retention and separation from endogenous substances as well as a sufficient signal-to-noise ratio. Ion-exchange chromatography [2] is often not compatible with MS detection due to the high ionic strength of the mobile phases required to elute nucleotides, particularly triphosphates, and these molecules are not retained under conventional reversed-phase conditions. Ion-suppression HPLC [9], capillary electrophoresis [10] and ion-pairing HPLC [11–24] have been employed to circumvent the poor retention problem.

In ion-pairing HPLC, trialkyl and tetraalkyl ammonium salts are usually added to the HPLC mobile phase. These positively charged ammonium salts form ‘‘ion-pairs’’ with the negatively charged nucleotides. The ‘‘ion-pairs’’, being more lipophilic, are retained on the HPLC columns. A number of trialkyl and tetraalkyl ammonium salts [11,12,15–17] have been employed including tetraethylammonium salts, tetrabutylammonium salts and tripropylammonium salts. Although these ion-pairing agents provide excellent chromatographic resolution, most of them are incompatible with electrospray ionization mass spectrometry (ESI-MS) due to their relatively non-volatile nature and the high concentration (~100 m*M*) needed for separation [17]. Several approaches have been adopted to overcome this problem including use of volatile ion-pairing agents [18–20], post-column removal of ion-pairing agents with an in-line membrane [21], post-column addition of sheath liquid [22] and splitting of the eluent flow [23].

Another challenge for the rapid monitoring of the nucleoside and nucleotide analogs is the low detection limits needed because the compounds are generally present at sub-micromolar levels in cells [1,2]. The negative ion mode of ESI-MS has been

commonly employed for detection of the nucleotides and oligonucleotides [16–20,22,23]. However, we observed that negative ion mode ESI does not provide enough sensitivity for detecting CBV-TP in cell extracts. Moreover, Ziagen has a poor MS response with negative ion mode ESI-MS, which prohibits simultaneous and sensitive quantitation of this nucleoside analog and its anabolites by HPLC–MS.

In this paper, we report a method using ion-pairing HPLC–MS–MS to simultaneously quantify Ziagen and its phosphorylated metabolites in cell extracts at nanomolar levels with positive ion mode ESI-MS.

2. Experimental

2.1. Chemicals

ATP and dGTP were purchased from Sigma (St. Louis, MO, USA). *N′*,*N′*-dimethylhexylamine (DMHA) and tetrapropylammonium hydroxide (TPA) were purchased from Aldrich (Milwaukee, WI, USA). Tetrabutylammonium bromide (TBA) was purchased from Fluka (Buchs, Switzerland). HPLC-grade methanol and water were purchased from EMI Science (Gibbstown, NJ, USA). Formic acid was purchased from GFS Chemicals (Columbus, OH, USA). Ziagen, Ziagen-MP, CBV-TP, CBV-DP and CBV-MP were prepared at Glaxo Wellcome. Stock solutions of ATP, Ziagen, Ziagen-MP, CBV-MP, CBV-DP and CBV-TP were made up at 1 m*M* in water and stored at −20°C until use.

2.2. Liquid chromatography

The HPLC separation was performed on a HP1100 liquid chromatography system equipped with a thermostated autosampler and a heated column compartment (Agilent Technologies, Palo Alto, CA, USA). A reversed-phase HPLC column [Luna, $C_8(2)$, 5 μm particles, 50×2.0 mm; Phenomenex, Torrance, CA, USA] was used. The autosampler was set at 10°C to minimize chemical hydrolysis of the nucleotides. The LC column was maintained at 25°C. The mobile phases consisted of 20 m*M* DMHA at pH 7 (A) and methanol–water (80:20) (B). The analytes were eluted with a gradient program: 30% B from 0 to 2 min; 30–80% B from 2 to 6 min; 80% B from 6 to 9 min; 80–30% B from 9 to 9.5 min at a flow-rate of 0.2 ml/min. This gradient was used for all experiments unless specified otherwise. The volume of sample injected onto the column was 10 μl.

2.3. Ion-trap mass spectrometry

Initial method development was conducted on a Bruker Esquire ion-trap mass spectrometer (Bruker-Daltoniks, Bremen, Germany). Solutions of ATP containing various ion-pairing agents were introduced into a pneumatically-assisted ESI interface from either the HPLC pump or an infusion pump (Harvard Apparatus, South Natick, MA, USA). Nitrogen was used as the nebulizer gas at 30 p.s.i. and the drying gas at a flow-rate of 10 l/min at 350°C (1 p.s.i.=6894.76 Pa). The mass scan range was from m/z of 140 to 600 u. In order to investigate the effect of different ion-pairing agents on MS sensitivity, infusion experiments with 50 μ*M* ATP in methanol–water (1:1) solution in the absence or in the presence of various ion-pairing agents at pH 7 were performed [24]. Full-scan mass spectra were acquired on the ion-trap mass spectrometer. Analyses were performed in either the negative or positive ion modes of ESI.

2.4. Tandem mass spectrometry

All HPLC–MS–MS experiments were conducted on a Sciex API-300 triple quadrupole mass spectrometer fitted with a Turbo Ionspray interface (PE-Sciex, Toronto, Canada). The flow-rates of curtain, nebulizing and collision gases were at settings of 12, 10 and 1, respectively. The turbospray gas was nitrogen at 8 l/min at 400°C. The nebulizer potential, orifice potential and collision energy were set at 4000 V, 50 V and 33 eV, respectively. All experiments were conducted in the positive ion mode. Other MS–MS parameters were optimized to achieve optimal sensitivity for the quantitation of CBV-TP. For the quantitation experiments, the mass spectrometer was scanned in the multiple reaction monitoring (MRM) mode. The transitions monitored were m/z 287→191 for Ziagen, m/z 367→191 for Ziagen-MP, m/z 508→136 for ATP, m/z 488→152 for CBV-TP, m/z 408→152 for CBV-DP and m/z

328→152 for CBV-MP. The data were collected by PE-Sciex software and processed by MacQuan (version 1.6). Post-analysis of the data was performed using Microsoft Excel (Redmond, WA, USA).

2.5. Sample preparation

Human peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers were extracted with 60% methanol at a density of $50 \cdot 10^6$ cells per ml. Following incubation with 10 μ*M* Ziagen, HepG2 2.2.15 cell extracts were prepared as described elsewhere [25] and stored at −80°C until analyzed. Calculation of drug concentrations in the HepG2 cells was based on an intracellular water volume of 2.6 μl per 10^6 cells [25].

For calibration, a series of human PBMC extract standards were prepared having Ziagen, CBV-MP, CBV-DP and CBV-TP at concentrations of 0.025, 0.05, 0.1, 0.5, 1, 2.5 and 5 μ*M*. dGTP standards were prepared in water with the same calibration range. Ziagen-MP standards were prepared by spiking into human PBMC extracts at concentrations of 0.010, 0.025, 0.05, 0.1 and 0.5 μ*M*. Linear regression analysis gave calibration curves that were used to calculate the analyte concentrations.

For precision analysis, the samples were prepared at 0.5 and 2.5 μ*M*. Six replicates of each concentration were assayed to obtain statistical data on the assay reproducibility.

3. Results and discussion

3.1. Selection of an ion-pairing agent

Due to the presence of multiple negative charges on the phosphate group, the endogenous nucleotides such as ATP as well as nucleotide analogs such as the anabolites of Ziagen are not expected to retain on conventional reversed-phase HPLC columns. Thus, the first criterion in selecting an appropriate ion-pairing agent was to ensure that the nucleotides were retained and separated on a reversed-phase HPLC column. We investigated several trialkyl and tetra-alkylammonium salts as ion-paring agents by HPLC–UV–Vis. The nucleotides were retained on the column when either 20 m*M* DMHA or 50 μ*M* TPA was added to the mobile phase A. The retention of the nucleotides can be explained by the formation of ''ion-pairs'' between the positively charged ion-pairing agents and negatively charged nucleotides [26]. These ion-pairs mask the charges on the nucleotides, which in turn facilitates their retention on the hydrophobic column. Another possibility is the adsorption of the positively charged ion-pairing agent onto the surface of the packing material, thus, rendering it similar to ion-exchange columns [27]. Also, the ion-pairing agent might be adsorbed to the column, making a more polar stationary phase and then retaining the nucleotide analytes.

Mass spectrometric detection of the nucleotides was examined because mass spectrometric (especially MS–MS) detection provides structural identification, specificity and better sensitivity than UV–Vis. In ESI-MS, a low concentration of volatile salts has been used to avoid decreasing MS sensitivity [24]. Therefore, the challenge was to select an ion-pairing agent that did not cause interference in mass spectrometric detection of the nucleotides. Since nucleotides have commonly been detected by negative ion mode ESI-MS due to the presence of the negatively charged phosphate group [17–19], negative ion mode ESI-MS was initially used. Fig. 2 shows the negative ion mass spectra of ATP without ion-pairing agent (A), with 10 m*M* DMHA at pH 7 (B) and 10 m*M* TPA at pH 7 (C). When 10 m*M* DMHA was employed, there was no significant difference in ion intensity of $[ATP-H]^-$ between the absence of and the presence of ion-pairing agent (Fig. 2A and B). However, a 100-fold decrease in $[ATP-H]^-$ peak height was observed when 10 m*M* TPA was used (Fig. 2C), compared to the signal without TPA (Fig. 2A).

Furthermore, we have investigated the effect of the ion-pairing agent (DMHA) on MS sensitivity in the positive mode of ionization. As shown in Fig. 3A, there was no significant ion signal of $[ATP+H]^+$ detected in the absence of DMHA. However, in the presence of DMHA, a strong signal of the $[ATP+H]^+$ was observed (Fig. 3B). The use of DMHA enhanced the protonation of the nucleotide (see Section 3.2 for detailed discussion) and made the detection of the $[ATP+H]^+$ species possible. The ability to detect nucleotides with positive ESI pro-

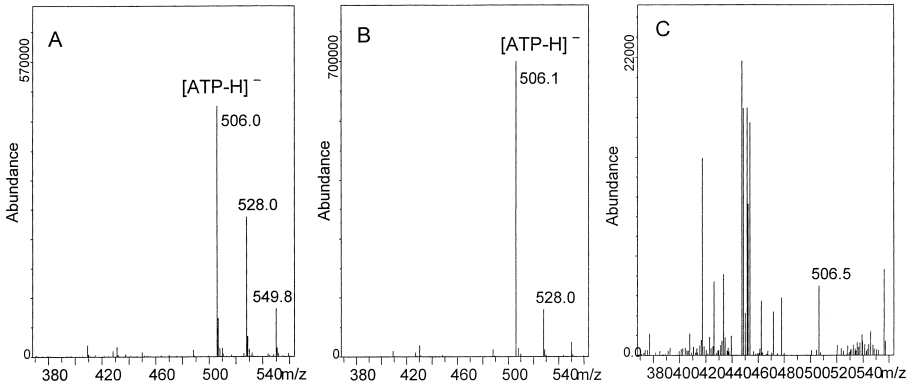

Fig. 2. (A) MS spectrum of ATP in negative ESI mode in the absence of ion-pairing agent. The experiment was done by infusing 50 μ*M* ATP in 50% methanol. The *m*/*z* 506 ion was $[ATP-H]^-$, the *m*/*z* 528 ion was $[ATP+Na-2H]^-$ and the *m*/*z* 550 was $[ATP+2Na-3H]^-$. (B) MS spectrum of ATP in negative ESI mode in the presence of DMHA. The experiment was done by infusing 50 μ*M* ATP in a 10 m*M* DMHA at pH 7 containing 50% methanol. (C) MS spectrum of ATP in negative ESI mode in the presence of TPA. The experiment was done by infusing 50 μ*M* ATP in a 10 m*M* TPA at pH 7 containing 50% methanol. All the experiments were done on the Esquire ion-trap mass spectrometer.

vided the potential for simultaneous determination of Ziagen and its anabolites, as Ziagen has a better MS response in the positive ion mode of ESI-MS than in the negative ion mode.

DMHA was found to cause minimal interference with mass spectrometric detection of nucleotides and to provide good sensitivity at positive ion mode of ESI-MS as well as adequate chromatographic separation (see Section 3.3 for further discussion), it was therefore selected as the ion-pairing agent for all subsequent experiments.

3.2. Detection of nucleotides by positive ion mode ESI-MS

Nucleotides have been commonly detected by negative ion mode ESI-MS due to the presence of one or more charges on the phosphate group. However, negative ion mode ESI-MS detection of the nucleotides did not provide the detection limits for the CBV-TP needed to support drug metabolism and pharmacokinetics studies (unpublished data). Furthermore, Ziagen, which is an uncharged nucleoside,

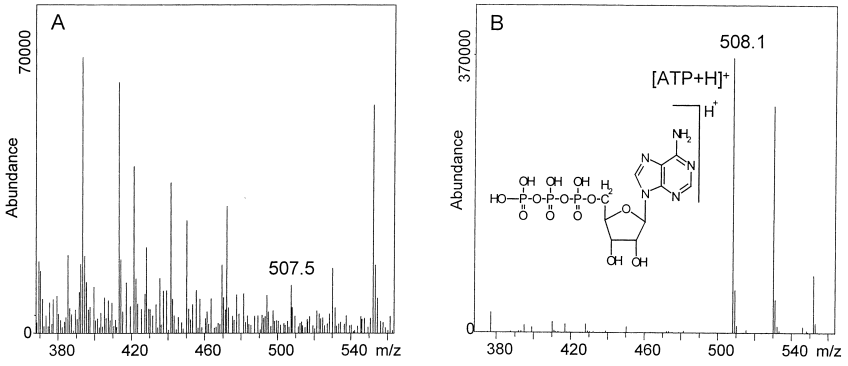

Fig. 3. (A) MS spectrum of ATP in positive ESI mode in the absence of ion-pairing agent. The experiment was done by infusing 50 μ*M* ATP in 50% methanol. (B) MS spectrum of ATP in positive ESI mode in the presence of DMHA. The experiment was done by infusing 50 μ*M* ATP in 10 m*M* DMHA at pH 7 and 50% methanol. All the experiments were done on the Esquire ion-trap mass spectrometer.

has a poor MS response in negative ion mode ESI-MS. This proves to be problematic for the simultaneous determination of nucleoside and nucleotide analogs. As mentioned in the previous section, we observed a strong $[ATP+H]^+$ signal in the presence of DMHA in the positive ion mode of ESI-MS while there was no detectable $[ATP+H]^+$ signal in the absence of DMHA. This was unexpected for the detection of nucleotides with ESI, even though protonated oligonucleotides have been observed with matrix-assisted laser desorption ionization (MALDI) and fast atom bombardment (FAB) [28–30]. However, the mechanism of the electrospray ionization in the presence of DMHA remains to be investigated. The protonation (and the overall positive charges) was likely due to the addition of the proton onto the nucleobase [31,32]. In the presence of DMHA, the negative charges on the phosphate group were masked and the nucleobase (adenine in the case of ATP) became protonated. The basicity of the nucleobase must have played a crucial role in this ionization scheme. This is evidenced by the fact that thymine-containing nucleosides and nucleotides such as dTTP, with thymine being a weaker base, had more efficient ionization in the negative ion mode of ESI-MS than in the positive ion mode of ESI-MS.

The effect of DMHA on MS sensitivity in both positive and negative ion modes of ESI-MS–MS was then investigated. CBV-TP was used as the test compound. The transition m/z 488→152 was chosen for positive ion mode ESI while the transition m/z 486→159 was chosen for negative ion mode ESI. These two transitions were chosen because they provided the base ion peaks for the tandem mass spectrometric experiments. During infusion experiments, the m/z 488→152 transition (for positive ion mode ESI-MS) was found to have better sensitivity than the m/z 486→159 transition (for negative ion mode ESI-MS). This was further supported by the quantitative analysis of CBV-TP with HPLC–MS–MS. Equal amounts (50 pmol) of CBV-TP were analyzed by ion-pairing HPLC–MS–MS with either positive ion mode ESI-MS (Fig. 4A) or negative ion mode ESI-MS (Fig. 4B). The MS–MS conditions were optimized for the corresponding transitions. It was found that positive ion mode ESI-MS provides stronger signal intensity compared to that for negative ion mode ESI-MS (Fig. 4).

In addition to better sensitivity and specificity, positive ion mode ESI allowed simultaneous determination of nucleoside and nucleotide analogs in the presence of DMHA.

3.3. Simultaneous quantitation of nucleosides and nucleotides

3.3.1. Separation of Ziagen and its phosphorylated metabolites by ion-pairing HPLC–MS–MS

Having selected an ion-pairing agent that allows retention and detection of nucleotides, the method was applied to separate a mixture of Ziagen, CBV-TP, CBV-DP, CBV-MP and ATP prepared in water. A representative chromatogram is shown in Fig. 5. All five compounds were well separated. This demonstrates the power of ion-pairing HPLC–MS–MS as a two-dimensional separation technique over those of either ion-pairing HPLC or MS–MS. This point is illustrated in the separation of Ziagen, ATP and CBV-TP (Fig. 5). These three compounds were not baseline resolved chromatographically. However, they gave distinctive transitions, m/z 287→191, m/z 508→136 and m/z 488→152 for Ziagen, ATP and CBV-TP, respectively. Each of those transitions resulted from cleavage of the respective glycosidic linkages. MRM was performed on these transitions. Clearly, these three compounds were resolved with MRM even though they were not resolved chromatographically.

In contrast, CBV-TP, CBV-DP and CBV-MP had to be separated chromatographically to avoid cross interference in mass spectrometric detection. This is because MS–MS data indicated that fragmentation of $[CBV\text{-}TP+H]^+$ at m/z of 488 generated fragment ions at m/z of 408 and 328, resulting from the loss of one or two terminal phosphoryl groups. It therefore caused cross interference for the analysis of CBV-DP and CBV-MP because the fragment ions from $[CBV\text{-}TP+H]^+$ had the same masses as those of $[CBV\text{-}DP+H]^+$ and $[CBV\text{-}MP+H]^+$. Thus, by combining ion-pairing HPLC and tandem mass spectrometry, all five compounds were separated in less than 13 min.

3.3.2. Endogenous nucleotides

Numerous endogenous nucleotides are present in cells [5]. These nucleotides can be broadly classified

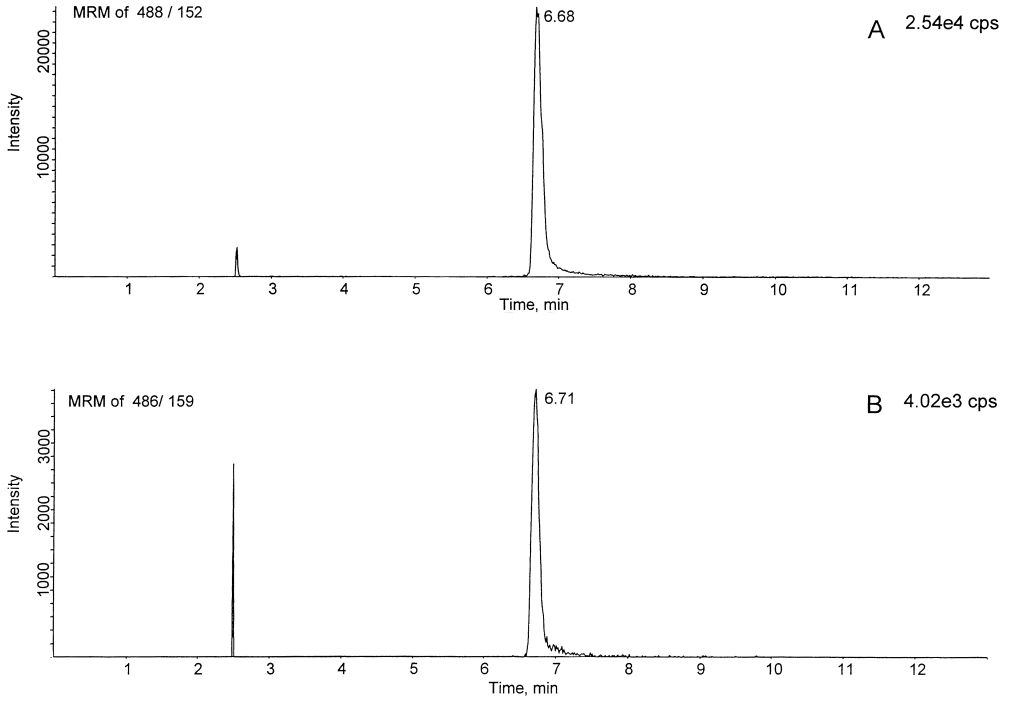

Fig. 4. LC–MS–MS chromatograms of CBV-TP in (A) positive ESI and (B) negative ESI modes.

into two groups, 2′-deoxyribonucleotides (5′-mono-, di-, triphosphate derivatives of dAdo, dGuo, dCyd and dThd) and ribonucleotides (5′-mono-, di-, triphosphate derivatives of Ado, Guo, Cyd and Urd). Since the exogenous nucleotide analogs compete with the endogenous deoxyribonucleoside triphosphates during reverse transcription, monitoring their concentrations is important in understanding their mechanism of action. For example, CBV-TP is known to be the competitor of dGTP [1]. The ratio of CBV-TP to dGTP can provide insight on the exposure or response of the drug. Thus, it is important to be able to quantify the amount of both dGTP and CBV-TP. As shown in Fig. 6, the same method could be used to analyze dGTP. The transition chosen was m/z 508→152. Since these endogenous nucleotides gave distinctive fragmentation patterns, by selecting the appropriate MRM transition (for example, dATP had a transition of 492→136, dCTP had 468→112), these nucleotides can be separated and quantified if needed. For pyrimidine nucleotides such as dTTP and UTP, negative ion mode ESI-MS is needed for detection as discussed previously.

Ribonucleoside triphosphates are present in cells at relatively high concentrations [5], thus, separating them (especially ATP, which can be present at millimolar concentrations) from the nucleotide analogs is necessary to avoid interference. As demonstrated in the previous section, ATP could be separated from the nucleoside and nucleotide analogs by ion-pairing HPLC–MS–MS (Fig. 5).

3.3.3. Quantitative analysis of Ziagen, Ziagen-MP, CBV-TP, CBV-DP, CBV-MP and dGTP in cell extracts

The method development for the simultaneous analysis of Ziagen, CBV-TP, CBV-DP and CBV-MP was conducted using PBMC extracts. Calibration curves for Ziagen, CBV-TP, CBV-DP and CBV-MP in the range of 25 nM to 5 μM were prepared by plotting the MRM ion peak responses vs. the corresponding concentrations of each analyte. Linear response ($r^2 \geq 0.99$) was obtained for all four ana-

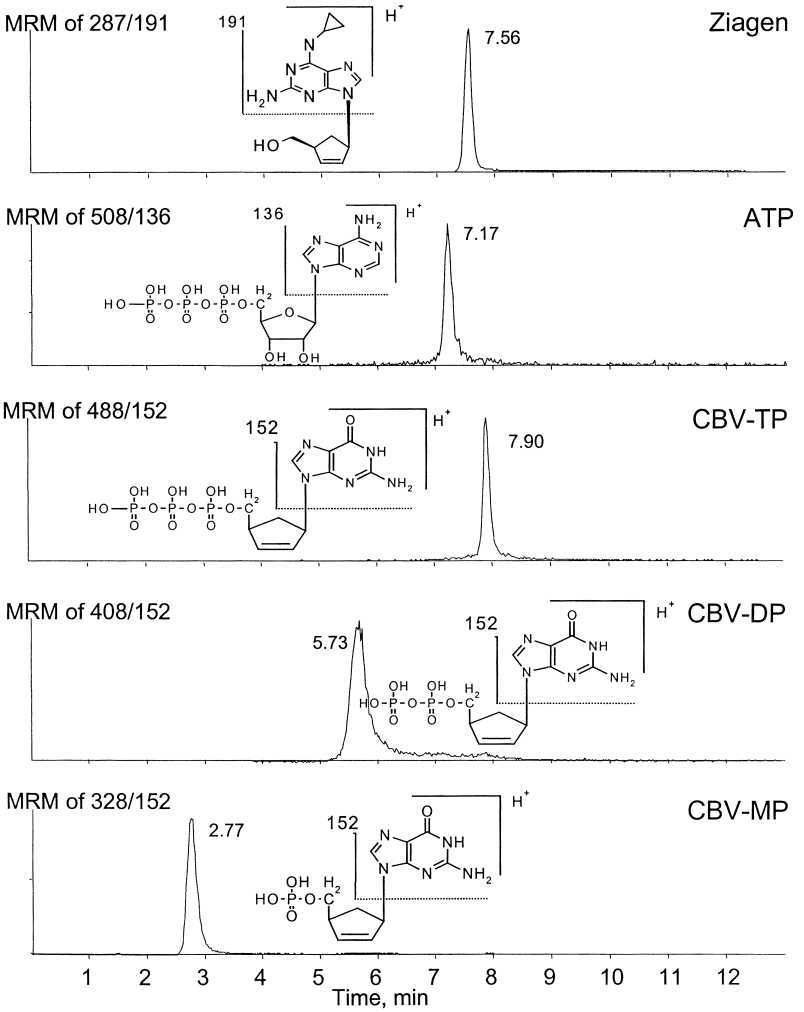

Fig. 5. LC–MS–MS chromatograms of Ziagen, ATP, CBV-TP, CBV-DP and CBV-MP. The gradient employed was 0–2 min: 30% B; 2–6 min: 30–60% B; 6–8 min: 60% B; 8–8.5 min: 60–20% B.

lytes. Quality control (QC) analyses were performed at concentrations of 0.5 and 2.5 μ*M*, and the results are summarized in Table 1. The QC samples were analyzed at the beginning, middle and end of each sample batch to control the instrument sensitivity variation. The results in Table 1 indicate that the assay provides good accuracy and precision. The limits of detection for the analytes were better than 25 n*M*. CBV-TP at a concentration of 25 n*M* (0.25 pmol injected) was analyzed by the HPLC–MS–MS method and the signal-to-noise ratio obtained was greater than 3 (Fig. 7).

Because conversion of Ziagen to Ziagen-MP is the first step in the metabolism of Ziagen to its active metabolite CBV-TP [2], determination of the concentration of Ziagen-MP may provide valuable information on the bioactivation kinetics. Thus, the assay was extended to the analysis of Ziagen-MP. A separate set of experiment was performed on human PBMC extracts spiked with Ziagen-MP. Linearity ranged from 10 n*M* to 0.5 μ*M*. The relative standard deviation (RSD) and accuracy of the QC samples ($n=2$ at 0.5 μ*M*) were 2.4% and 14%, respectively.

The method was applied to the analysis of extracts

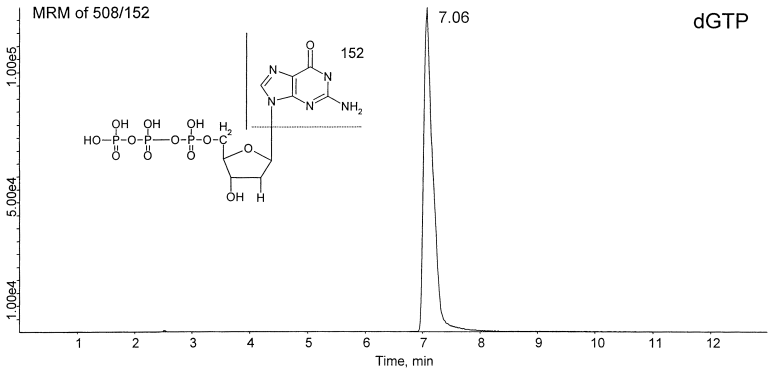

Fig. 6. LC–MS–MS chromatogram of dGTP. The gradient employed was the same as that of Fig. 5.

Table 1
Accuracy and precision data of simultaneous analysis of human PBMC extracts spiked with Ziagen, CBV-MP, CBV-DP and CBV-TP

Compound	Spiked concentration					
	2.5 μ*M*			0.5 μ*M*		
	Mean±SD[a]	Relative error (%)	RSD[a] (%)	Mean±SD[a]	Relative error (%)	RSD[a] (%)
Ziagen	2.72±0.19	8.7	7.0	0.44±0.01	−11.4	2.9
CBV-MP	2.80±0.18	11.8	6.4	0.45±0.01	−9.5	2.2
CBV-DP	2.66±0.13	6.5	5.0	0.47±0.02	−6.2	4.8
CBV-TP	2.76±0.10	10.3	3.7	0.43±0.03	−15.0	7.9

[a] $n=6$.

of HepG2 2.2.15 cells treated with 10 μ*M* of Ziagen. Fig. 8 shows the LC–MS–MS chromatograms of these extracts. Ziagen and CBV-TP in the treated cells were quantified by using calibration curves ranging from 25 n*M* to 5 μ*M*. The concentrations of Ziagen and CBV-TP in these cell extracts were 2.7 μ*M* ($n=2$, RSD=4%) and 0.056 μ*M* ($n=3$, RSD=6.4%), respectively, and corresponded to intracellular levels of 10.4 and 0.22 μ*M*. The CBV-TP level determined by this method was comparable to the levels seen in another study conducted under similar conditions using radiolabeled material and analyzed by the conventional anion-exchange method (0.26 μ*M*) (unpublished results).

Fig. 8 shows mass chromatograms obtained from the analysis of HepG2 2.2.15 cell extracts. Note that retention times of the analytes were different from those in Fig. 5 due to slightly different HPLC gradient program was used (see figure legends for detailed HPLC conditions). It is interesting to note that CBV-DP was detected at a retention time of 6.35 min. It was not, however, quantified because its

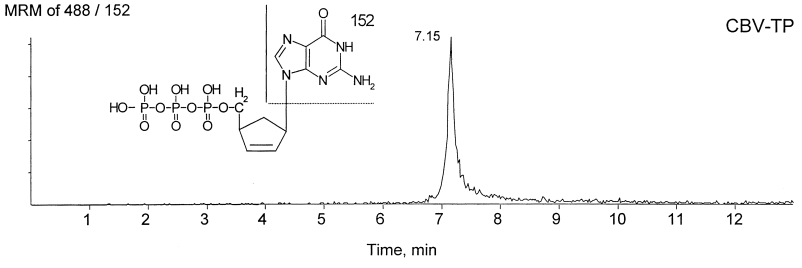

Fig. 7. LC–MS–MS chromatogram of CBV-TP spiked in human PBMC extracts at 25 n*M*.

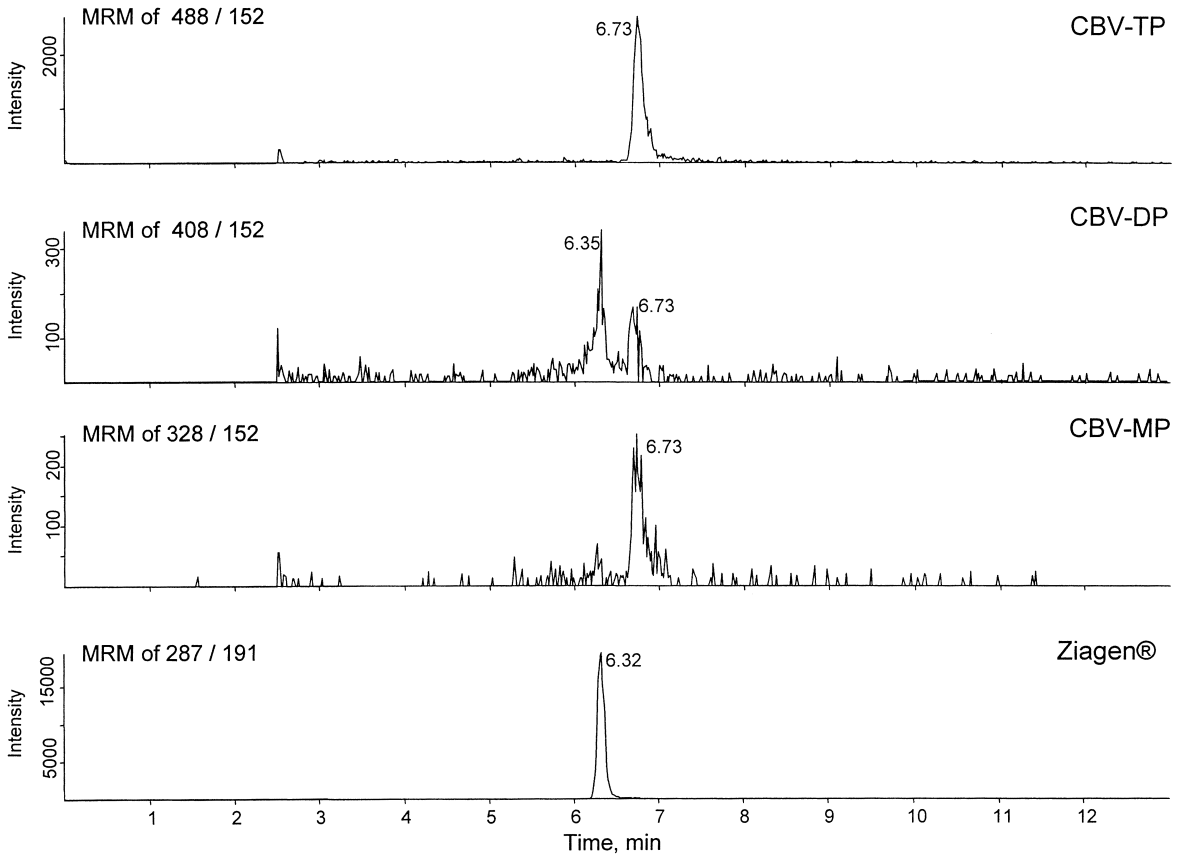

Fig. 8. LC–MS–MS chromatograms of extracts of HepG2 2.2.15 cells treated with 10 μ*M* Ziagen. The gradient employed was 0–2 min: 20% B; 2–5 min: 20–80% B; 5–7 min: 80% B; 7–7.5 min: 80–20% B.

concentration was below the calibration range. The peak at 6.73 min resulted from the fragmentation of CBV-TP (see the discussion in Section 3.1). CBV-MP and Ziagen-MP were not detected in the cell extract samples. For quality control, an extract from untreated cells was also analyzed. No major peaks of the nucleoside or nucleotide analogs were detected in the untreated cell extracts.

The extracts were also analyzed for two endogenous nucleotides, ATP and dGTP in a separate run. The dGTP concentration in the cell extracts was calculated to be 0.27 μ*M* (n=2, RSD=8.9%). An intense peak of ATP was detected. Its concentration, however, was not quantified.

In conclusion, an ion-pairing HPLC–MS–MS method was developed to separate negatively charged nucleotides. It has been successfully applied to analyze Ziagen and its anabolites in both human PBMC extracts and extracts of Ziagen-treated HepG2 2.2.15 cells. Preliminary data showed that the method can also be applied to other nucleoside and nucleotide analogs such as lamivudine (3TC), 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T), 3′-azido-3′-deoxythymidine (AZT), lamivudine triphosphate (3TC-TP), 2′,3′-didehydro-2′,3′-dideoxythymidine triphosphate (d4T-TP) and 3′-azido-3′-deoxythymidine triphosphate (AZT-TP). In the case of thymidine analogs such as AZT, AZT-TP, d4T and d4T-TP, however, negative ion mode ESI provided better MS sensitivity for the nucleosides and their nucleotide analogs. The more efficient ionization in the negative ion mode of ESI was probably caused by the fact that thymine was a weaker base, which made the deprotonation process more efficient than

the protonation process. Work is in progress to apply the method to these nucleoside and nucleotide analogs and other endogenous and exogenous nucleotides.

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