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Ultra sensitive method for the determination of 9-(2-phosphonylmethoxyethyl)adenine in human serum by liquid chromatography–tandem mass spectrometry

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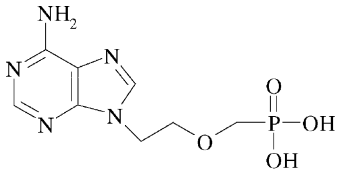
Abstract

An ultra sensitive method for the direct measurement of 9-(2-phosphonylmethoxyethyl)adenine (PMEA), an antiviral agent for hepatitis B, in human serum using high performance liquid chromatography/tandem mass spectrometry (LC–MS/MS) has been developed. This method involves the addition of [^{13}C]PMEA (contains 5 ^{13}C) as internal standard, the purification and enrichment by a MCX solid phase extraction (SPE) cartridge, and quantitative analysis using LC–MS/MS. The MS/MS is selected to monitor the *m*/*z* 272 → 134 and *m*/*z* 277 → *m*/*z* 139 transitions for PMEA and [^{13}C]PMEA, respectively, using negative electrospray ionization. The MS/MS response is linear over a concentration of 0.1–10 ng/ml with a lower limit of quantitation (LLOQ) of 0.1 ng/ml. The mean inter-assay accuracy (%Bias) for quality control (QC) at 0.1, 0.25, 1.0, and 10 ng/ml are 10, 1.6, −0.8, and 0.0%, respectively. The mean inter-assay precision (%CV) for the corresponding QCs is 3.9, 3.8, 5.3, and 3.4%, respectively. The method has been used to determine PMEA concentration in human serum following a single oral administration of a PMEA pro-drug at dose of 10 and 30 mg.

Keywords: 9-(2-Phosphonylmethoxyethyl)adenine

1. Introduction

9-(2-Phosphonylmethoxyethyl)adenine (PMEA), has broad-spectrum activity against herpes-, retro-, and hepadnaviruses [1–4]. However, PMEA exhibited low oral bioavailability in both animals [5,6] and humans [7]. It also presented significant concern of kidney toxicity [8].



PMEA

Adefovir dipivoxil (Hepsera, Gilead Science) is an oral prodrug of PMEA. Preliminary clinical trials showed that it decreased serum HBV DNA and alanine amino transaminase levels at doses of 5, 30 and 60 mg per day in both HBeAg-positive and HBeAg-negative patients with chronic hepatitis B [9,10]. However, there was dose-limiting kidney toxicity for Hepsera. Incidence of serum creatinine increase (0.3–0.5 mg/dl from baseline) was significantly higher at 30 mg than 10 mg [6]. Therefore a 10 mg was recommended [11] which probably is a sub-optimal dose.

Previously, Augustijns et al. [12] developed a HPLC/UV method for the measurement of PMEA with the assay sensitivity of 1.0 μg/ml. Cundy et al. [7] reported a HPLC/fluorescence method with a detection limit of 250 ng/ml. Naesens et al. [13] had modified the HPLC/fluorescence method and improved the detection limit down to 50 ng/ml. Subsequent refinements in the assay methodology by Cundy [14] led to a validated limit of quantitation in human serum of 25 ng/ml. However, all these methods do not have sufficient sensitivity to evaluate pharmacokinetics of PMEA following oral administration of adefovir dipivoxil at the approved dose of 10 mg once daily. Therefore, an ultra-sensitive LC–MS/MS method for the determination of PMEA in human serum with much improved sensitivity

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was developed and validated. The method has been used to determine the concentration of PMEA in human serum following oral dosing of a PMEA prodrug.

2. Experimental

2.1. Chemicals and materials

PMEA was supplied by Metabasis Inc. (San Diego, CA). [^{13}C]PMEA, 9-(2-phosphonylmethoxyethyl)-)-(2,4,5,6,8-[^{13}C])adenine, was synthesized at Moravek Biochemicals, Inc. (Brea, CA). *N*,*N*-Dimethylhexylamine (DMHA) was purchased from Sigma (St. Louis, MO). Acetic acid, acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Ammonium acetate was purchased from ICN Pharmaceuticals (Costa Mesa, CA). MCX solid phase extraction (SPE) cartridges were purchased from Waters Corporation (Milford, MA).

2.2. Drug administration and sample collection

Six subjects received 10 mg of prodrug and 14 subjects received 30 mg of the same prodrug in the study. Serum samples were collected at 0 (pre-treatment), 1, 2, 3, 4, 8, 12, 24, 48, and 72 h.

2.3. Sample extraction

A solution of [^{13}C]PMEA internal standard (10 ng/ml in water, 25 μl) and 500 μl 0.1 N HCl solution were added to human serum (500 μl). The sample was vortexed briefly for 1 min. The MCX cartridges were conditioned by 1 ml methanol and 1 ml water. The samples were partitioned onto MCX cartridge and the cartridge was rinsed with 1 ml 0.1 N HCl solution followed by 1 ml methanol. Analyte was eluted twice with 150 μl of 5% ammonium hydroxide in 80/20 (v/v) acetonitrile/water solutions each. The eluent (300 μl) was collected into a clean tube (13 mm × 100 mm) and evaporated to dryness using a TurboVap evaporator (15 psi N_2, 25 °C). The sample was reconstituted in 100 μl of DMHA/acetic acid/acetonitrile/water (1/0.5/80/18.5%, v/v/v/v). The final extract was analyzed by LC–MS/MS for the quantitation of PMEA in human serum.

2.4. Preparation of calibration and quality control (QC) samples

Calibration standards were prepared at seven concentration levels ranged from 0.1 to 10 ng/ml by spiking PMEA standard spiking solutions into human serum blanks (Bioreclamation Inc., Hicksville, NY), followed by sample preparation described below. The concentrations of QC samples are 0.1, 0.25, 1.0, and 10 ng/ml, respectively. QC samples were prepared by spiking the QC solution into human serum blank (5%, v/v) and were stored at −80 °C until analysis.

Table 1
HPLC gradient conditions for LC–MS/MS analysis of PMEA

Time (min)	*A* (%)	*B* (%)
0.0	90.0	10.0
0.5	90.0	10.0
3.5	50.0	50.0
5.0	50.0	50.0
5.1	90.0	10.0
6.0	90.0	10.0

A: 0.1% (v) acetic acid in water; *B*: 0.1% (v) acetic acid in acetonitrile.

Table 2
MS/MS conditions for PMEA analysis

Mass spectrometer	API4000			
Interface	TurboIon spray			
Polarity	Negative			
Scan type	MRM			
Resolution	Q1—unit[a], Q3—low[b]			
Curtain gas (CUR)	10			
Collision gas (CAD)	5			
IonSpray voltage (IS)	−1500			
Temperature (TEM)	650 °C			
Ion source gas 1 (GS1)	60			
Ion source gas 2 (GS2)	60			
Solvent split ratio	None			
Compound	Mass to charge ratio (*m*/*z*)		Time (ms)	Collision energy (V)
	Q1	Q3		
PMEA	272	134	150	−30
[^{13}C]PMEA	277	139	150	−30

[a] Unit: FWHM is 0.7 ± 0.1 amu.
[b] Low: 0.04 amu offset drop from unit.

2.5. LC–MS/MS conditions

A Cohesive high throughput liquid chromatographic (HTLC) system (Franklin, MA) equipped with a API4000 mass spectrometer (Applied Biosystems, Foster City, CA) was used to validate the method and conduct the sample analysis. An XDB-C_8 analytical column (4.6 mm × 150 mm, 5 μm, Agilent) was used for the analysis to avoid tailing interactions with residual silanols. DMHA was utilized as an ion paring reagent for PMEA, which is a very polar compound to achieve better chromatography. The LC and MS/MS conditions are summarized in Tables 1 and 2. The product ion scan spectra of PMEA, and [^{13}C]PMEA are presented in Fig. 1.

3. Results

The analysis of blank human serum indicated no interference of endogenous compounds with PMEA in the final

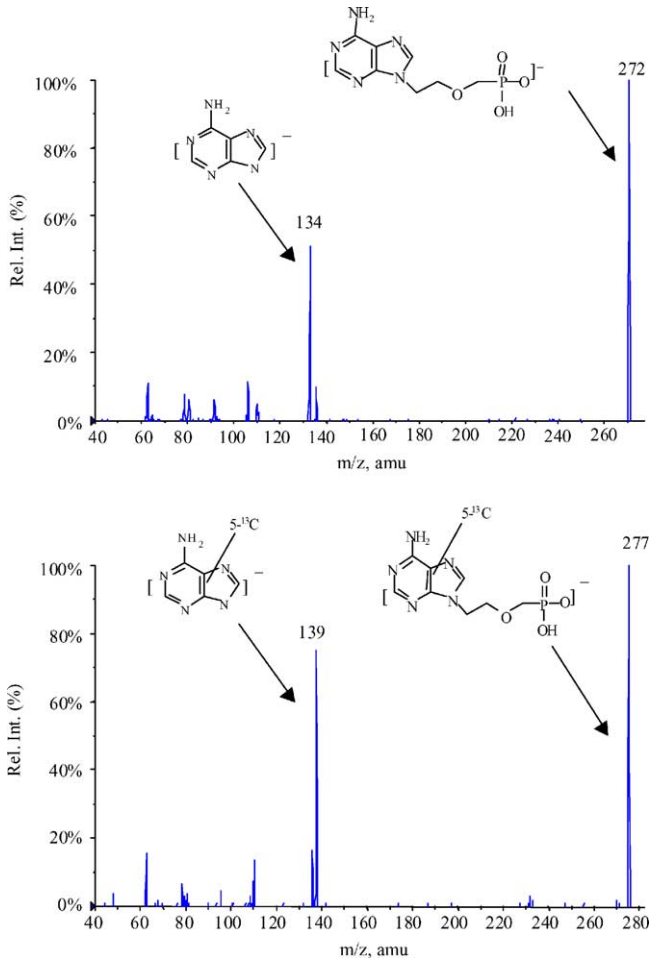

Fig. 1. Product ion scan spectra of (a) PMEA and (b) [^{13}C]PMEA.

extract. Typical chromatograms of a blank human serum extract and LLOQ sample are presented in Figs. 2 and 3. The calibration curve was obtained by fitting the ratio of integrated mass peak area of PMEA to that of internal standard against a range of concentrations (0.1–10 ng/ml) of added PMEA using 1/x linear regression plots. The correlation coefficients (r) were greater than 0.998 for all of the calibration curve determinations during the method validation. The mean bias ranges from −2.1 to 5% and the mean reproducibility (CV%) ranges from 1.2 to 4.9% (Table 3). A representative calibration curve of PMEA is shown in Fig. 4.

The method provided satisfactory detection sensitivity with a LLOQ at 0.1 ng/ml for PMEA, which was established by a small coefficient of variation (3.9%) and an acceptable bias (10%). Accuracy and Precision are determined based on LLOQ, low-, mid-, and high-QC samples. The inter-assay shows that mean accuracy ranged from −0.8 to 10% with their nominal values while %CV varying between 3.4 and 5.3% (Table 4). These values are well within the acceptance criteria recommended by the FDA guideline (21 CFR 58).

Overnight bench stability, freeze and thaw stability as well as human serum extract stability were examined. Overnight bench stability of the final extract was determined by reanalyzing the QC samples form day 1 method validation set with day 2 validation set. The mean recovery of the LOQ-QC was 0.110 ng/ml, indicating that PMEA was stable during storage.

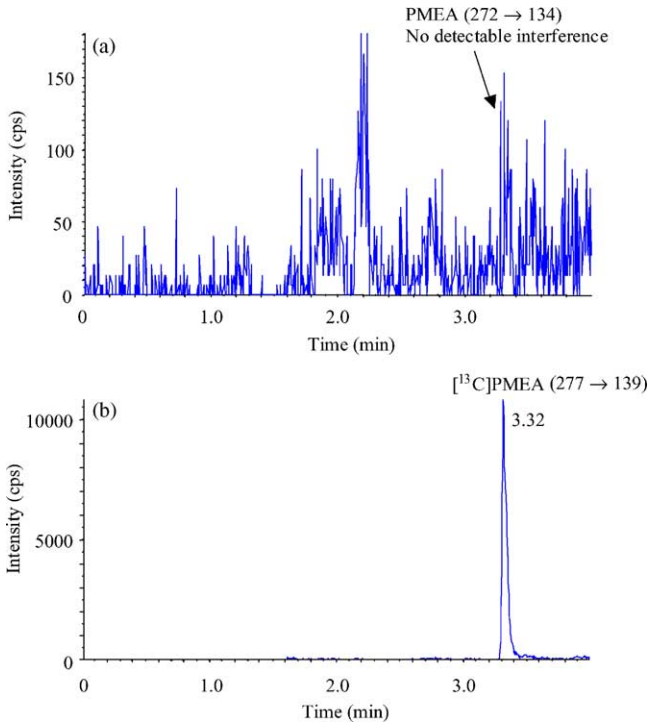

Fig. 2. Typical extracted ion chromatograms of human serum control blank extract (a) PMEA (b) [^{13}C]PMEA.

Freeze and thaw stability was determined by freezing and thawing the low- and high-QC samples for three

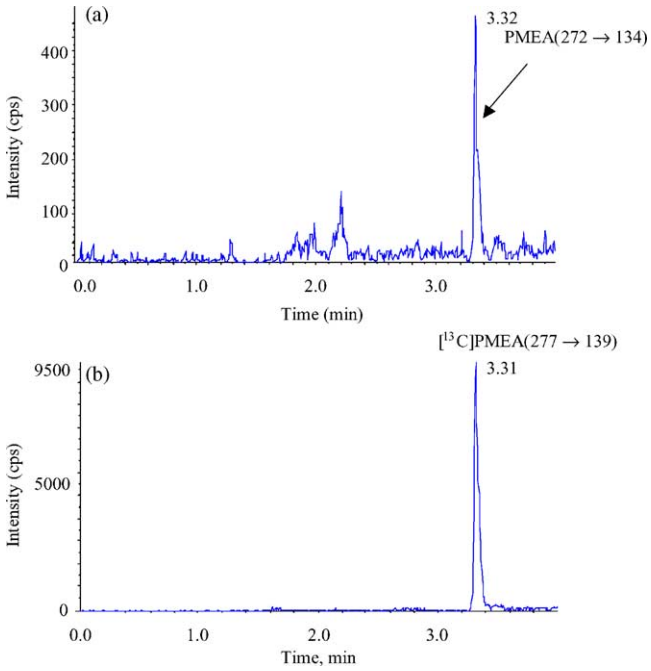

Fig. 3. Typical extracted ion chromatograms of human serum LLOQ (100 pg/ml) extract (a) PMEA (b) [^{13}C]PMEA.

Table 3
Calibration curve analytical results for PMEA in human serum

Set	Curve #	Concentration of standards (ng/ml)						
		0.100	0.250	0.500	1.00	2.00	5.00	10.0
1	1	0.110	0.236	0.504	0.953	1.98	4.93	10.1
	2	0.109	0.241	0.488	0.951	2.02	4.98	10.1
	3	0.103	0.249	0.481	1.01	1.96	5.12	9.93
	4	0.108	0.238	0.501	0.974	1.97	4.94	10.1
2	1	0.108	0.258	0.487	0.968	1.93	4.77	10.3
	2	0.0967	0.270	0.478	1.03	2.00	4.75	10.2
	3	0.108	0.256	0.484	0.918	1.99	5.09	10.0
	4	0.0981	0.258	0.505	1.03	1.86	5.08	10.0
Mean		0.105	0.251	0.491	0.979	1.96	4.96	10.1
S.D.		0.00519	0.0118	0.0107	0.0405	0.0498	0.141	0.118
%CV		4.9	4.7	2.2	4.1	2.5	2.8	1.2
%Bias		5.0	0.4	−1.8	−2.1	−2.0	−0.8	1.0
n		8	8	8	8	8	8	8

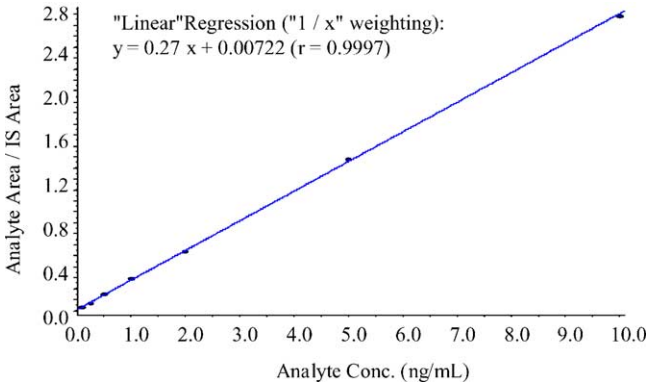

Fig. 4. Typical calibration curve for the determination of PMEA in human serum.

cycles before sample processing with at least 12 h interval between each cycle. The mean recovery of the low- and high-QC samples were 92.0 and 94.1% indicating that PMEA was also stable after three freeze and thaw cycles.

The LC–MS/MS method was used to analyze serum levels of PMEA in man following single oral administration of a PMEA prodrug. Serum concentrations of PMEA after oral dose of 10 and 30 mg of the prodrug are illustrated in Fig. 5. Using this analytical method with much improved sensitivity, we were able to measure concentration of PMEA up to 96 h (last time point for serum sample collection) from all subjects after 30 mg dose of prodrug, up to 24 h from all subject after 10 mg dose, up to 48 h from four out of six subjects after 10 mg dose and up to

Table 4
Quality control analytical results for PMEA in human serum

Set	Parameter	LOQ-QC 0.100 ng/ml	Low-QC 0.250 ng/ml	Mid-QC 1.00 ng/ml	High-QC 10.0 ng/ml
1		0.109	0.264	0.978	10.1
		0.107	0.247	0.973	10.2
		0.115	0.250	0.931	9.81
	Mean	0.110	0.254	0.961	10.0
	S.D.	0.00416	0.00907	0.0258	0.203
	%CV	3.8	3.6	2.7	2.0
	%Bias	10.0	1.6	−3.9	0.0
	n	3	3	3	3
2		0.108	0.255	0.981	10.1
		0.116	0.267	0.998	9.51
		0.106	0.243	1.09	10.5
	Mean	0.110	0.255	1.02	10.0
	S.D.	0.00529	0.0120	0.0586	0.498
	%CV	4.8	4.7	5.7	5.0
	%Bias	10.0	2.0	2.0	0.0
	n	3	3	3	3
Overall statistics	Mean	0.110	0.254	0.992	10.0
	S.D.	0.00426	0.00954	0.0530	0.340
	%CV	3.9	3.8	5.3	3.4
	%Bias	10.0	1.6	−0.8	0.0
	n	6	6	6	6

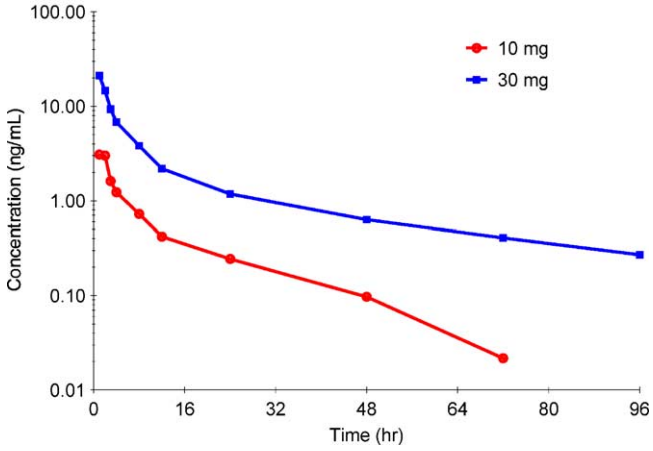

Fig. 5. Serum concentrations of PMEA following single oral dosing of a prodrug of PMEA at 10 and 30 mg.

72 h from one out of six subjects after 10 mg of dose of prodrug.

4. Discussion

Adefovir dipivoxil was readily absorbed after oral administration to mice [13], rats [15], dogs [16] and man [17]. Adefovir dipivoxil and its monoester (mono-POM PMEA) were not observed in plasma, suggesting rapid hydrolysis of the prodrug to adefovir (PMEA) before reaching the systemic circulation. Therefore pharmacokinetics of PMEA was evaluated in animals and man following oral dosing of the prodrug. Due to limitation of the assay sensitivity, plasma levels of PMEA were analyzed only up to 8 h in man after oral dose of 125 mg, 12 h in rats following oral dose of 10 mg/kg and 24 h in dogs after oral dose of 10 mg-PMEA equivalent/kg. Recently a C_{max} of 18–20 ng/ml with a last measurable concentration of about 1.5 ng/ml at 36 h in man following an oral dose of 10 mg of adefovir dipivoxil was reported. However, the analytical method for the determination of PMEA in human serum in this study has not yet been reported. To properly evaluate pharmacokinetics of a drug, the analytical method needs to be able to measure at least seven half-lives. Therefore we urgently need to develop an analytical method, which enable us to measure serum level as low as 0.15 ng/ml.

We have successfully developed and validated a very sensitive LC–MS/MS method for the determination of PMEA in human serum with a LLOQ of 0.1 ng/ml. This was achieved through (1) larger serum volume, (2) SPE enrichment, (3) HPLC separation, (4) LC–MS/MS monitoring and (5) stable isotope internal standard. SPE technique provides better sample cleaning and trace enrichment compare to protein precipitation extraction. HPLC provides good separation between PMEA and endogenous components from human serum. LC–MS/MS monitoring greatly improved the selectivity. This method used isotope compound of PMEA as internal standard. The [^{13}C]PMEA (internal standard) has five ^{13}C in the adenine ring of PMEA. Its MS/MS transition at 277 → 139 is 5 amu higher than that of PMEA at 272 → 134. This mass difference eliminates the concern of cross talk in the triple quadruple mass spectrometer and greatly improved the selectivity and sensitivity of the method. The sensitivity was also improved by utilizing of API 4000 mass spectrometer. Typical with signal-to-noise ratio is greater than 10 as presented in Fig. 3.

Using this analytical method with much improved sensitivity, we were able to measure concentration of PMEA up to 96 h (last time point for serum sample collection) from all subjects after 30 mg dose of prodrug, up to 24 h from all subject after 10 mg dose, up to 48 h from four out of six subjects after 10 mg dose and up to 72 h from one out of six subjects after 10 mg of dose of prodrug.

In conclusion, an ultra sensitive method has been developed to determine the PMEA concentration in human serum samples. The calibration curves are linear over a concentration range of 0.1–10 ng/ml with a LOQ at 0.1 ng/ml. The use of the [^{13}C]PMEA internal standard improves the accuracy and precision of the method. Because the low LOQ achieved in this method, the concentration of PMEA can be monitored up to the last serum sample collection time (96 h) at 30 mg dose and up to 48 h after 10 mg of dose of the prodrug.

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