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Development and validation of a sensitive LC–MS/MS method for the determination of adefovir in human serum and urine

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Abstract

A sensitive and selective liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed and validated for the determination of adefovir (PMEA) in human serum and urine. The analyte was separated on a Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size) by isocratic elution with methanol–water–formic acid (20:80:0.1, v/v/v) at a flow rate of 0.6 ml/min, and analyzed by mass spectrometry in multiple reaction-monitoring mode. The precursor-to-product ion transitions of *m/z* 274 → 162 and *m/z* 226 → 135 were used to measure and quantify the analyte and internal standard (I.S.), respectively. The weighted ($1/x^2$) calibration curve was linear over serum concentration range 1.25–160.00 ng/ml and urine concentration range 0.05–8.00 μg/ml, with a correlation coefficient (*r*) of 0.9992 and 0.9978, respectively. The lower limit of quantification in human serum was 1.25 ng/ml. The inter- and intra-day precisions (R.S.D.%) in both serum and urine were lower than 8.64%, the mean method accuracies and recoveries from spiked serum samples at three concentrations ranged from 96.3 to 102.0% and 56.5 to 59.3%, respectively. The serum extract was stable when stored for 24 h. The developed method was successfully applied to determine PMEA in human serum and urine, and proved suitable to clinical pharmacokinetic study.

Keywords: Adefovir; LC–MS/MS; Pharmacokinetics; Human

1. Introduction

Adefovir [9-(2-phosphonylmethoxyethyl)adenine, PMEA, Fig. 1], a phosphonic acid derivative of adenine, has broad-spectrum activity against human immunodeficiency virus (HIV), herpes viruses, hepatitis B viruses (HBV) and adenoviruses [1], and its bis-pivaloyloxymethyl ester (bis-POM-PMEA, Adefovir Dipivoxil, ADV) with higher oral bioavailability has been used for the treatment of chronic hepatitis B in adults with evidence of HBV replication. ADV is rapidly hydrolysed to PMEA in gastrointestinal tract after oral administration [2]. PMEA is transported into cells and converted to active PMEA diphosphate with intracellular half-life of 12–36 h [3]. The mean maximum concentration (C_{max}) of PMEA in plasma was 18.4 ± 6.26 ng/ml, and the steady urinary recovery was approximately 45.3% [4], following oral administration of single dose of ADV 10 mg to patients with chronic hepatitis B or healthy subjects.

PMEA in plasma, serum or urine were previously determined by HPLC with fluorescence detection after being derivatized with chloroacetaldehyde, further more, an ion-pair agent was needed to be added to mobile phase [5,6]. The pretreatment of samples was fairly tedious and time-consuming, and the method was not sensitive enough to evaluate the pharmacokinetics of PMEA following the approved oral dose of ADV (10 mg once daily). Zhao et al. [7] and Liu et al. [8] developed a sensitive LC–MS/MS method for the determination of PMEA in human plasma and serum, with the internal standard (I.S.) 9[(*R*)-2-(phosphonomethoxy)propyl]adenine (PMPA) and [^{13}C]PMEA, respectively. Unfortunately, the two substances used as I.S. are difficult to obtain. Acyclovir (Fig. 1) is somewhat similar to PMEA in chemical structure and readily available, so it is selected as I.S. to develop a sensitive and accurate LC–MS/MS method for the determination of PMEA in human serum following oral administration of ADV.

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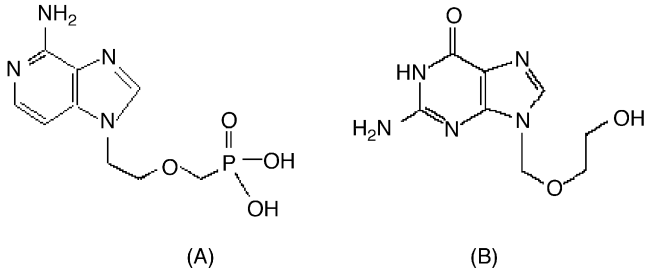

Fig. 1. Chemical structures of: (A) adefovir and (B) acyclovir.

2. Experimental

2.1. Chemicals and reagents

PMEA standard (99.2%) was provided by Qilu Pharmaceutical Company (Jinan, China). The internal standard, acyclovir (99.7%), was commercially obtained from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of chromatographic grade was purchased from Tedia Company (USA); formic acid was analytical grade and the water was deionized and double-distilled.

2.2. LC–MS/MS instrument and conditions

The HPLC system (Agilent 1100 Series, Agilent Technologies, USA), equipped with a binary pump, a vacuum degasser, a thermostatted column compartment and an autosampler (140 vials capacity), were used for solvent and sample delivery. Chromatographic separation was performed on a Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size, Beijing Dikma Company, China). Isocratic elution of the analyte from the column was achieved with the mobile phase consisting of methanol–water–formic acid (20:80:0.1, v/v/v) at a flow rate of 0.6 ml/min. The column temperature was maintained at 25 °C.

An API 4000 triple–quadrupole mass spectrometer (AB Sciex Instruments, USA) with electrospray ionization (ESI) source was used for mass detection and analysis. Mass spectrometric analysis was performed in positive ion mode and set-up in multiple reaction-monitoring (MRM) mode. Nitrogen served as nebulizer (GS1), auxiliary (GS2), curtain (CRU) and collision gas (CAD) in the API 4000. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on ANALYST™ 1.3 version software.

2.3. Preparation of standard and quality control samples

A stock solution of 800 μg/ml PMEA was prepared by dissolving the accurately weighted drug in water. Working solutions of PMEA were obtained by step-wise dilution of the stock solution. Internal standard stock solution (1000 ng/ml) was prepared in water, with further dilution of 500 ng/ml for a working solution. All these solutions were stored at 4 °C before use.

Calibration standards were prepared by spiking blank human serum or urine with 50 μl of each appropriate stock standard solution. The concentration range for serum and urine calibration curve was 1.25–160.00 ng/ml and 0.05–8.00 μg/ml, respectively.

Table 1
Main working parameters of tandem mass-spectrometer

Parameter	Value
Turbo ionspray temperature, TEM (°C)	450
Dwell time per transition (ms)	200
Ion source gas 1, GS1 (kPa)	413.40
Ion source gas 2, GS2 (kPa)	310.05
Curtain gas, CUR (kPa)	103.35
Collision gas, CAD (kPa)	34.45
Ion spray voltage, IS (V)	5400
Entrance potential, EP (V)	14
Declustering potential, DP (V)	70 (analyte) and 37 (I.S.)
Collision energy, CE (V)	37 (analyte) and 35 (I.S.)
Collision cell exit potential, CEP (V)	15 (analyte) and 14 (I.S.)
Mode of analysis	Positive
Ion transition for PMEA (*m/z*)	274 → 162
Ion transition for I.S. (*m/z*)	226 → 135

Quality control (QC) samples at three different concentrations (2.50, 20.00 and 80.00 ng/ml for serum, and 0.10, 1.00 and 4.00 μg/ml for urine) were also prepared with blank human serum or urine, but spiked with independently prepared stock standard solutions.

2.4. Drug administration and sample collection

An open-label, randomized triple cross-over study with a washout period of 1 week was performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Nine healthy male subjects with mean age of 23.1 ± 1.5 years and body weight of 65 ± 7 kg were enrolled and randomized into 3 groups to receive 10, 20 or 40 mg of ADV tablets for pharmacokinetic study. After an overnight fasting (10 h), each subject received the scheduled dose with 250 ml of water. No food was allowed until 4 h after dose administration. Water intake was allowed after 2 h and low fat standard meals were provided at 4 and 10 h post-dose.

Blood sample (4 ml) was collected at 0 (pre-dose), 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 h post-dose. Serum was separated, decanted, frozen and stored at −80 °C for analysis.

Urine sample was collected prior to dosing and over the intervals 0–2, 2–4, 4–6, 6–8, 8–10, 10–12 and 12–24 h post-dose. The total volume of urine collected in each interval was recorded and 10 ml from which was centrifuged, decanted, frozen and stored at −80 °C for analysis.

2.5. Sample preparation

Serum sample (0.5 ml) was pipetted into a micro tube, and 50 μl of double-distilled water and 50 μl of I.S. solution were added and mixed, then 1.0 ml of methanol was added. The mixture was vortexed for 1 min, and centrifugated at 9500 × *g* for 5 min. The supernatant was transferred, evaporated to dryness and reconstituted with 200 μl of mobile phase, then 40 μl of which was injected for analysis.

Urine sample was centrifugated at 1520 × *g* for 5 min, and then 50 μl of which was diluted with 50 μl of double-distilled water and 0.9 ml of mobile phase. The mixture was vortexed for 1 min and 20 μl of which was injected for analysis.

3. Results and discussion

3.1. MS/MS optimization

The full-scan mass spectra of PMEA and I.S. and product ion mass spectra of $[M+H]^+$with MRM mode are shown in Figs. 2 and 3, respectively. PMEA and I.S. gave protonated parent ion $[M+H]^+$ at *m/z* 274 and *m/z* 226, and the fragment ions of the most significant intensity were observed at *m/z* 162 for PMEA and *m/z* 135 for I.S., respectively. So the mass transitions chosen for quantitation were *m/z* 274 → 162 for PMEA and *m/z* 226 → 135 for I.S.

The high-flow gas flow parameters were optimized by making successive flow injections while introducing mobile phase into the ionization source, and the instrument setting was adjusted to maximize the response for the analyte and I.S. by infusing their standard solutions with a syringe pump. The parameters presented in Table 1 are the results of this optimization.

3.2. HPLC separation and sample preparation

PMEA is insoluble in organic solvent such as chloroform, dichloromethane and acetic ether, so the serum protein was removed by precipitation. It was found that methanol and acetonitrile contributed to similar precipitation efficiency, but acetonitrile could lead to more matrix effects than methanol. When 10% trichloracetic acid was chosen for the protein precipitation, the peak area of analyte decreased notably 24 h post-extract. The reasons for this had not been confirmed, one possibility was that 10% trichloracetic might influence the stability of analyte, but no literature had ever been reported. Finally, methanol was selected as the protein precipitant, which was also used in mobile phase.

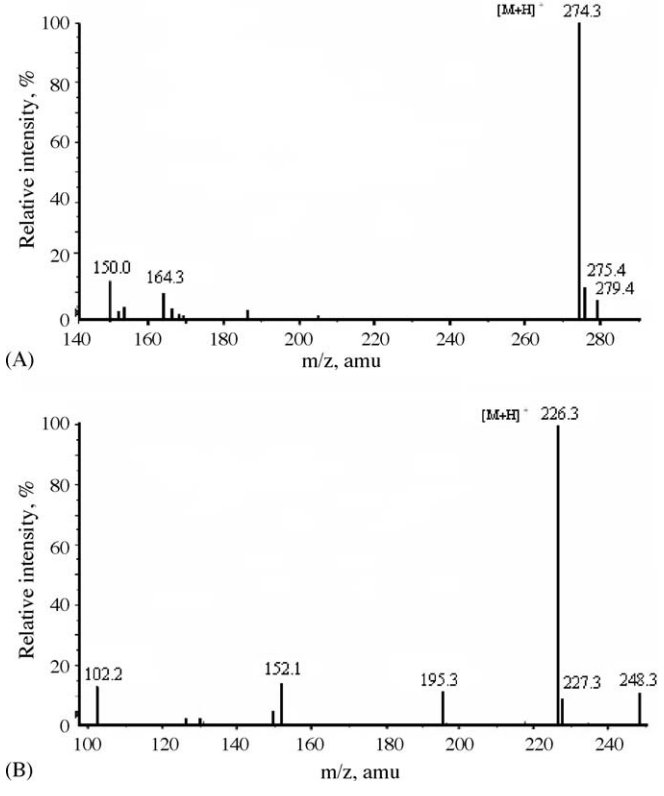

Fig. 2. Full scan mass spectra of: (A) PMEA and (B) I.S.

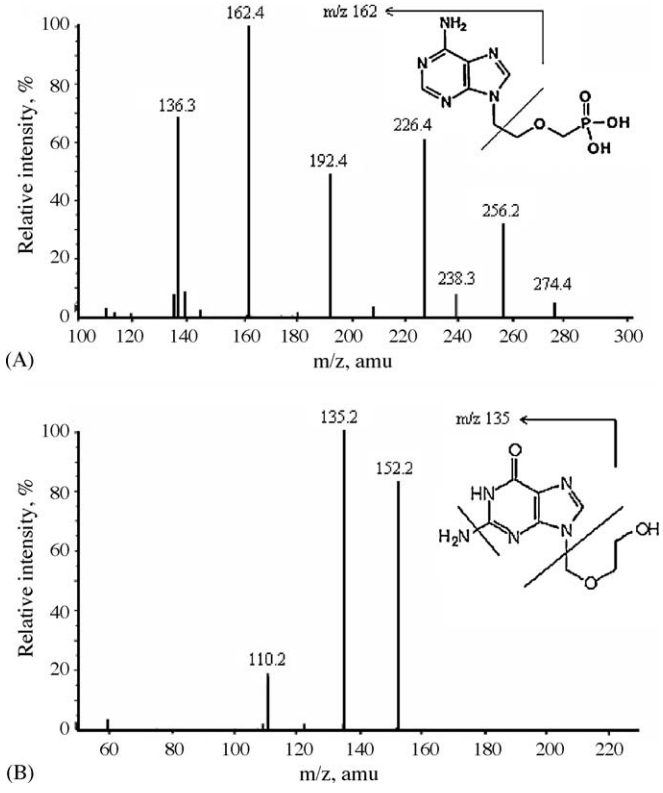

Fig. 3. Product ion mass spectra of: (A) PMEA and (B) I.S.

HPLC conditions were optimized to improve HPLC separation and enhance sensitivity. The composition of the mobile phase was optimized by varying percentages of methanol–water or acetonitrile–water. It was found that acetonitrile added to the mobile phase could lead to ionizing suppression and matrix effect on the quantification precision and accuracy, and the analytes could not be well separated from endogenous compounds with the mobile phase composed simply of methanol and water. The formic acid was found to be necessary to protonate the analytes and modify the peak shape. Finally methanol–water–formic acid (20:80:0.1, v/v/v) was adopted as mobile phase, due to the better separation, higher sensitivity and more stable MS signal. Under the optimized LC–MS/MS conditions, no interferences of endogenous compounds were found, and the retention time for PMEA and I.S. was 6.44 and 6.82 min, respectively.

It is necessary to use an I.S. to get high accuracy and deal with sample matrix effects when a mass spectrometer is used as the HPLC detector. PMPA and [^{13}C]PMEA were widely selected as I.S. for the determination of PMEA [5–8], but not readily available, so an alternative approach has been used. Internal standard

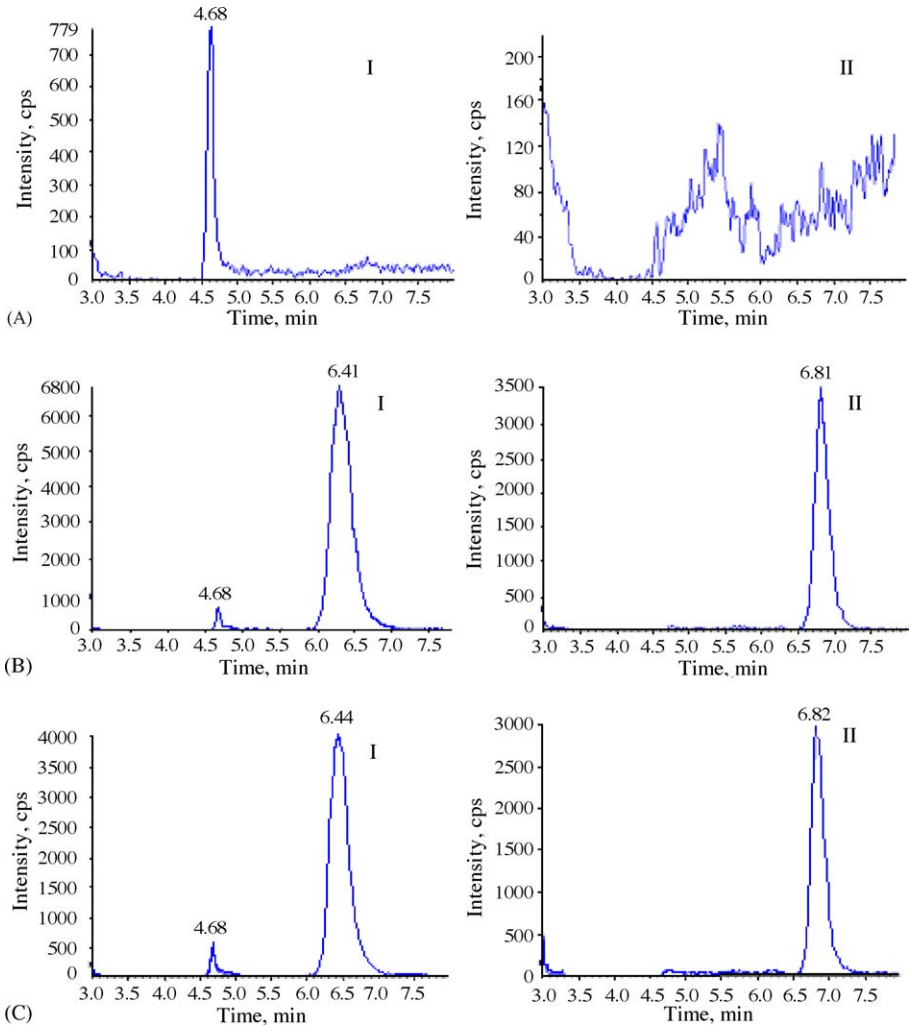

Fig. 4. Typical MRM chromatograms of: (A) blank serum; (B) blank serum spiked with 40 ng/ml of PMEA and 50 ng/ml of I.S.; (C) serum sample of a subject 3 h post-oral administration of ADV 20 mg. Peak I: PMEA; Peak II: I.S.

substance should match the chromatographic retention, recovery and ionization properties with the matrix of the analyte [9]. Acyclovir was found to fulfill these criteria sufficiently due to its similar chemical structure and chromatographic behavior to PMEA. It is the first time that acyclovir was adopted as the I.S. for the determination of PMEA, and which was proved to be feasible and acceptable.

Concentrations of PMEA in urine were fairly high because it is mainly excreted by kidney, and could be determined directly following simple dilution. The urine samples were thawed, centrifugated and 20-fold diluted with mobile phase, then determined under the same chromatographic conditions as mentioned above.

3.3. Method validation

3.3.1. Specificity

The analysis of blank human serum and urine indicated no interference of endogenous compounds with PMEA in the final extract. The specificity of the method was evaluated with individual serum and urine samples from six different sources. Typical chromatograms of blank serum, PMEA standard and volunteer serum sample are shown in Fig. 4, and those of urine samples are shown in Fig. 5.

3.3.2. Calibration curve

Calibration curves for serum were constructed by analyzing standard serum solutions, and the procedure was performed as described under "*Sample preparation*" except that the double-distilled water was replaced by PMEA standard solution. The mean of five independent measurements were taken into account. The peak area ratio (y) of PMEA to I.S. was measured and plotted against the concentrations (x) of PMEA spiked in blank serum. The calibration curve was linear over the concentration from 1.25 to 160.00 ng/ml. The weighted regression equation ($W = 1/x^2$) [10] of the calibration curve was $y = 0.0476x + 0.00685$ with a correlation coefficient (r) of 0.9992. The limit of detection (S/N = 3) was observed to be 1.0 ng/ml.

Calibration curves for urine were prepared at eight concentration levels by spiking serial PMEA standard solutions into human urine blanks, followed by "*Sample preparation*" except

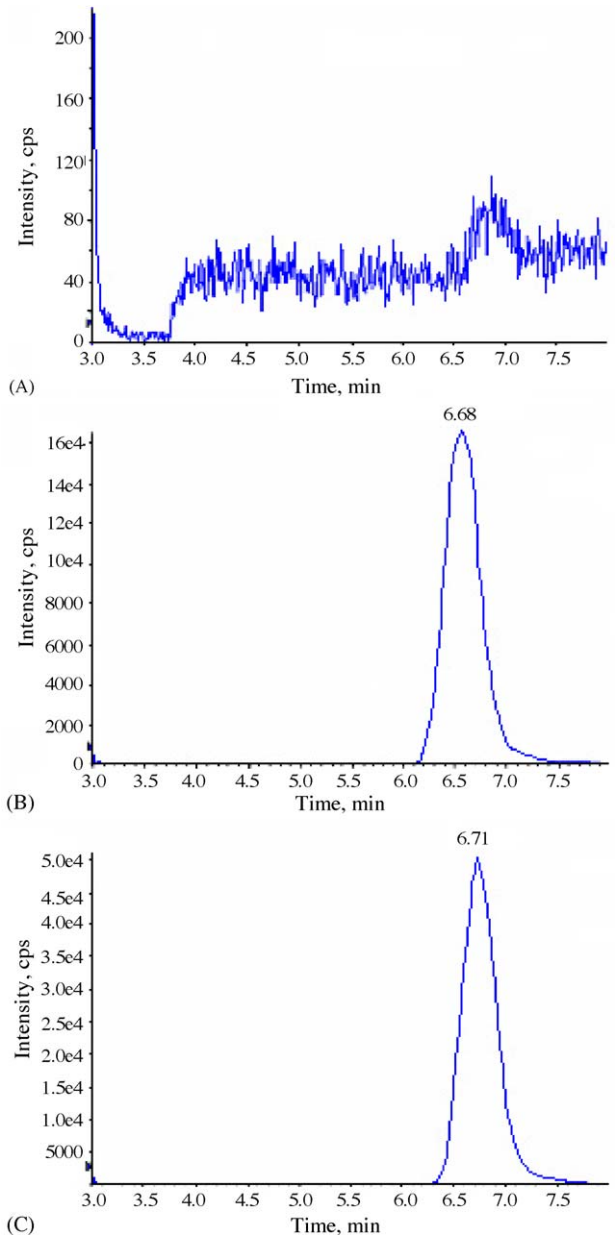

Fig. 5. Typical MRM chromatograms of: (A) blank urine; (B) blank urine spiked with 4 μg/ml of PMEA; (C) urine sample of a subject.

that the double-distilled water was replaced by PMEA standard solution. The peak area (y) of PMEA was measured and plotted against the concentrations (x) of PMEA spiked in blank urine. The calibration curve was linear over the concentration from 0.05 to 8.00 μg/ml with a weighted regression equation ($W = 1/x^2$) of $y = 4.44 \times 10^5 x + 1.95 \times 10^3$ ($r = 0.9978$).

3.3.3. Accuracy, precision and recovery

The precision and accuracy of the method was evaluated by analyzing serum and urine QC samples, prepared separately from calibration standards at a serum concentration of 2.50, 20.00 and 80.00 ng/ml, and urine concentration of 0.10, 1.00 and 4.00 μg/ml, respectively. Each concentration with five replicates was measured in one day or in five continuous days as described under "*Calibration curve*". The extraction recoveries were calculated by comparing the peak area of PMEA with those obtained by equal amounts in mobile phase solution. The accuracies were calculated by comparing the observed concentrations of PMEA from the regression equation with those spiked in serum. The intra- and inter-day precisions were determined by analyzing the concentrations of QC samples with five replicates on the same day and on separate days. These results are shown in Table 2, as indicate that the values are within acceptable range and the method is precise. The extraction recoveries of PMEA from serum were somewhat lower than those reported (more than 85%, by one-step protein precipitation with methanol) [11], which may be caused by the loss of analyte when it was transferred. It is important to be pointed out that no degradation of PMEA was found when it was evaporated to dryness, and no literature about this has ever been reported. In any case, the determination and quantification of analyte were not affected by the recovery.

3.3.4. Stability

Sparidans et al. [6] reported that PMEA in plasma appeared to be stable under all conditions tested, including storage at ambient temperature for 3 days, undergoing four freeze–thaw cycles and long-term storage at −20 °C for 4.5 months. Liu et al. [8] also reported that PMEA was stable in the final serum extract stored overnight and undergoing three freeze–thaw cycles. So in this study we only examined the stability of PMEA (2.50, 20.00 and 80.00 ng/ml) in the final serum extract when stored for 24 h. The mean recoveries of the low, medium and high-QC samples were

Table 2
Accuracy, precision and recovery of PMEA in serum and urine ($n = 5$)

Added amount	Intra-day		Inter-day		Accuracy (%)	Extraction recovery (%)
	Measured amount	Precision (R.S.D.%)	Measured amount	Precision (R.S.D.%)		
Serum sample (ng/ml)						
2.50	2.48	8.64	2.49	2.94	99.3	56.5
20.00	20.05	5.95	21.34	3.32	100.2	58.8
80.00	81.58	4.98	76.64	7.45	102.0	59.3
Urine sample (μg/ml)						
0.10	0.099	2.22	0.103	1.49	99.3	Not done
1.00	0.97	2.31	1.02	5.33	96.7	Not done
4.00	3.85	0.93	4.04	4.36	96.3	Not done

Table 3
Mean serum concentration of PMEA following single dose of ADV 10, 20 and 40 mg, respectively (mean ± S.D., $n = 9$)

Time/h	Mean serum concentration (ng/ml)		
	10 mg	20 mg	40 mg
0.50	13.62 ± 7.16	34.88 ± 16.00	53.41 ± 27.27
0.75	17.19 ± 5.98	40.82 ± 16.29	72.63 ± 27.45
1.00	20.12 ± 4.95	41.49 ± 12.39	78.57 ± 22.30
1.50	22.14 ± 5.90	39.50 ± 7.76	72.47 ± 17.00
2.00	19.69 ± 4.31	37.96 ± 6.94	67.64 ± 15.25
2.50	18.24 ± 3.85	34.79 ± 7.39	60.82 ± 12.83
3.00	17.27 ± 3.67	32.74 ± 7.30	54.87 ± 10.34
4.00	15.56 ± 3.08	29.81 ± 8.17	50.16 ± 10.14
6.00	14.27 ± 3.32	26.80 ± 7.47	41.96 ± 9.73
8.00	12.27 ± 2.70	22.66 ± 7.83	33.39 ± 11.94
12.00	8.36 ± 2.35	14.93 ± 5.07	23.11 ± 9.24
24.00	2.32 ± 0.72	4.73 ± 1.77	8.87 ± 2.90

92.3, 94.0 and 94.1%, respectively, indicating that PMEA was stable 24 h after final extract.

3.4. Applied in clinical pharmacokinetics

It is reported [5] that ADV could be rapidly converted to PMEA following oral administration, and no intact prodrug or monoester had been detected in blood. Therefore, the pharmacokinetic parameters of PMEA were evaluated following oral dose of the prodrug. The developed and validated LC–MS/MS method was used to analyze serum and urine concentrations of PMEA in men following single oral administration of ADV. Following oral administration of ADV 10, 20 and 40 mg, the mean serum concentration and main pharmacokinetic parameters of PMEA are presented in Tables 3 and 4, respectively. The mean serum concentration–time curves and cumulative urinary excretion–time curves of PMEA are shown in Figs. 6 and 7, respectively. Using this analytical method, we were able to measure the concentrations of PMEA up to 24 h (last time point for serum sample collection) from all subjects after 10 mg dose of ADV.

Table 4
Main pharmacokinetic parameters of PMEA following single dose of ADV 10, 20 and 40 mg, respectively (mean ± S.D., $n = 9$)

Parameter	10 mg-dose	20 mg-dose	40 mg-dose
K_a (h^{-1})	2.3 ± 0.8	1.9 ± 0.8	3.0 ± 1.1
$T_{1/2}$ (h^{-1})	6.8 ± 0.8	7.4 ± 1.1	7.7 ± 1.8
V_d/F (ml kg^{-1})	7.0 ± 2.0	7.4 ± 2.9	8.1 ± 2.1
CL/F (ml h kg^{-1})	0.63 ± 0.13	0.68 ± 0.12	0.91 ± 0.29
AUC_{0-24} (ng ml^{-1} h)	229 ± 37	434 ± 111	707 ± 186
$AUC_{0-\infty}$ (ng ml^{-1} h)	233 ± 37	438 ± 111	712 ± 185
MRT (h^{-1})	7.8 ± 0.8	7.7 ± 0.4	7.7 ± 0.6
C_{max} (ng/ml)	24 ± 5	48 ± 8	85 ± 19
T_{max} (h^{-1})	1.3 ± 0.4	1.1 ± 0.6	1.1 ± 0.4

K_a: absorption rate constant; $T_{1/2}$: terminal elimination half-life; V_d/F: apparent volume of distribution; CL/F: clearance; AUC: area under the concentration–time curve; MRT: mean residence time; C_{max}: peak concentration; T_{max}: time of peak concentration.

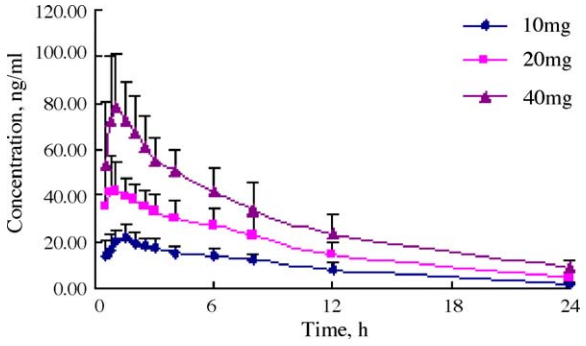

Fig. 6. Mean serum concentration–time curves of PMEA following single dose of ADV (mean ± S.D., $n = 9$).

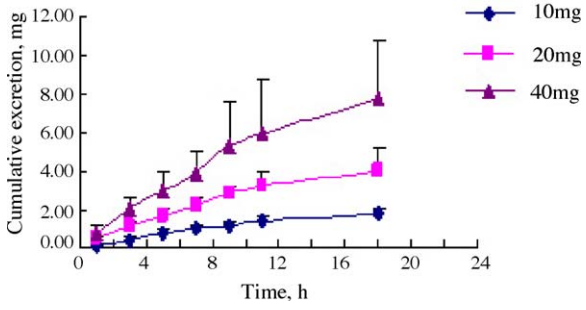

Fig. 7. Mean cumulative urinary excretion–time curves of PMEA following single dose of ADV (mean ± S.D., $n = 9$).

4. Conclusions

A sensitive and selective LC–MS/MS method was developed and validated for the determination of PMEA in human serum and urine. The separation of PMEA was performed on a Diamonsil C_{18} column with the mobile phase consisting of methanol–water–formic acid (20:80:0.1, v/v/v). The analyte was detected by tandem mass spectrometer operating in positive ionization mode. The ion transitions of m/z 274 → 162 and m/z 226 → 135 were used to quantify PMEA and I.S., respectively. A good linearity was obtained over a serum and urine concentration range of 1.25–160 ng/ml and 0.05–8.00 μg/ml, respectively. The lower limit of quantification in human serum was 1.25 ng/ml. The inter- and intra-day precisions (R.S.D.%) of the method were lower than 8.64%, the mean method accuracies and extraction recoveries at three concentrations ranged from 96.3 to 102.0% and 56.5 to 59.3%, respectively. The final serum extract was stable when stored for 24 h. Acyclovir was first used as I.S. for the determination of PMEA, which was proved to be simple, convenient and feasible. The developed method is proved suitable for human pharmacokinetic study of PMEA, and using which we can measure the concentration of PMEA up to 24 h after 10 mg oral dose of ADV.

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