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Improved and simplified liquid chromatographic assay for adefovir, a novel antiviral drug, in human plasma using derivatization with chloroacetaldehyde

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Abstract

A rapid and simplified chromatographic assay is reported for the quantification of adefovir (PMEA) utilizing derivatization with chloroacetaldehyde. Adefovir is isolated from plasma using protein precipitation with trichloroacetic acid; next, the fluorescent 1,N^6-etheno derivative is directly formed at 98°C in the buffered extract with chloroacetaldehyde. This derivative is analyzed using isocratic ion-pair liquid chromatography and fluorescence detection at 254 nm for excitation and 425 nm for emission. In the evaluated concentration range (10–1000 ng/ml) precisions ≤5% and accuracies between 95 and 117% were found, using a 0.2-ml volume of plasma. The lower limit of quantification is 10 ng/ml with a intra-assay precision of 16%. The currently reported bioanalytical method is 20–25-fold more sensitive than previously published assays.

Keywords: Derivatization, LC; Adefovir; Chloroacetaldehyde

1. Introduction

Adefovir [9-(2-phosphonylmethoxyethyl)adenine, PMEA, Fig. 1a], a phosphonic acid derivative of adenine, is a broad spectrum antiviral agent which can inhibit replication of HIV (human immunodeficiency virus), representatives of herpes viruses (cytomegalovirus), hepatitis B and adenoviruses [1,2]. Unlike natural nucleotides, an acyclic nucleotide phosphonate is resistant to phosphorolytic cleavages by (cellular) esterases. The limited oral availability of adefovir has been improved by using the bis-pivaloyloxymethyl ester of adefovir (bis-POM-PMEA, Preveon, Fig. 1b) as a pro-drug [3] in oral formulations. In this pro-drug the strongly polar phosphonic moiety of adefovir is shielded. Adefovir is the only intercellular metabolite identified after oral administration of this pro-drug thus far. Intracellularly, adefovir is phosphorylated to the active diphosphate of adefovir [4]. Presently, oral adefovir dipivoxyl is being evaluated in phase III clinical

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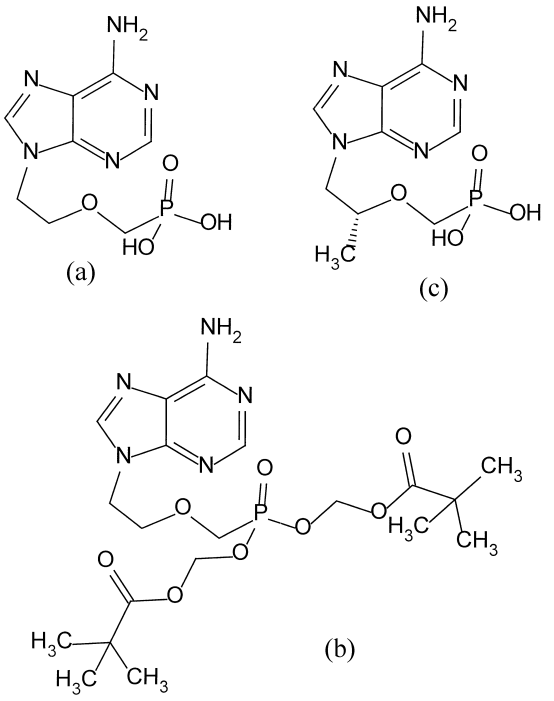

Fig. 1. Chemical structure of (a) adefovir, (b) adefovir dipivoxyl and (c) PMPA.

trials against HIV infection and in phase II trials against hepatitis B infection [5].

To support pharmacokinetic studies of adefovir (dipivoxyl) [6] bioanalytical assays for the quantification of this drug in plasma using a derivatization with chloroacetaldehyde (Fig. 2) and ion-pair liquid chromatography have been briefly described previously [3,4,7], as well as assays for adefovir in urine [4,8] using an analogous methodology. Unfortunately, however, lower limits of quantification (LLQs) for the reported assays in plasma, 200 [7] and 250 [4] ng/ml, respectively, are not only high for a fluorescent molecule, but also to high to measure clinically achieved concentrations of the drug in plasma. Furthermore, reported validation results are not complete and time-consuming pre-treatment procedures, i.e., protein precipitation combined with liquid–liquid extraction [7] or solid-phase extraction [4] prior to the derivatization or combined with evaporation of the aqueous samples before and after derivatization [3], have been reported. In addition, structural confirmation of the derivatization product of adefovir and chloroacetaldehyde has not been published yet and for two of the three methods [3,7] gradient elution was employed. Although an improved LLQ (25 ng/ml) was recently claimed for a non-published refinement of an existing assay [6], we investigated the possibilities for simplifying the sample pre-treatment and further improvement the sensitivity of the assay, using isocratic liquid chromatography. Complete validation results and the development of this improved assay are reported in this paper.

2. Experimental

2.1. Chemicals

Adefovir and 9-[(*R*)-2-(phosphonomethoxy)-propyl]adenine monohydrate (PMPA·H_2O, Fig. 1C) were both supplied by Gilead Sciences (Foster City, CA, USA). Acetonitrile (gradient grade) was provided by Biosolve (Valkenswaard, The Netherlands), chloroacetaldehyde (50%, w/w, in water) by Aldrich

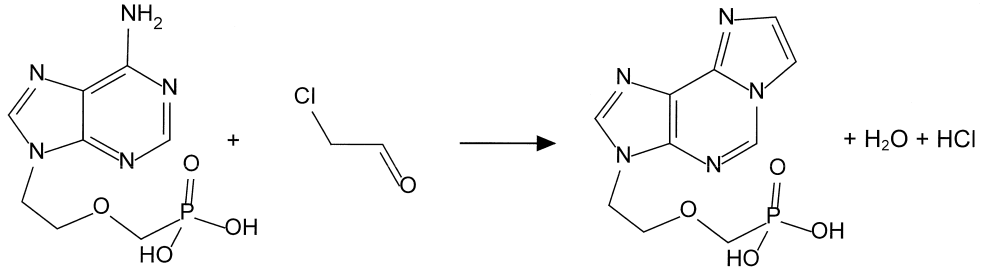

Fig. 2. Fluorogenic derivatization reaction of adefovir with chloroacetaldehyde; the reaction of PMPA is identical.

(Milwaukee, WI, USA) and phosphoric acid (85%, w/w, in water) by Baker (Deventer, The Netherlands). Trichloroacetic acid (TCA) and tetrabutylammonium-hydrogen sulfate were obtained from Janssen (Beerse, Belgium) and water was purified by reversed osmosis on a multi-laboratory scale. Other pharmaceutical agents, used for testing analytical interference, were obtained as pharmaceutical grade reference material or as a solution for injection; all other chemicals were of analytical grade from Merck (Darmstadt, Germany). Blank, drug-free human plasma was obtained at the Stichting Rode Kruis Bloedbank (Utrecht, The Netherlands)

2.2. Equipment

Chromatographic analyses were performed using the following configuration: A P580 isocratic pump (Gynkotek HPLC, Germering, Germany), a Basic+ Marathon autosampler (Spark Holland, Emmen, The Netherlands), equipped with a 7739-005 injection valve (Rheodyne, Cotati, CA, USA) with a 100-μl sample loop, and a FP-920 fluorescence detector (Jasco, Hachioji, Japan). The column was thermostated in a water bath and the temperature was controlled by a thermomix 1420 heating device (B. Braun, Melsungen, Germany). Chromatographic data were recorded on a Jotronics Pentium 166-32 Mb personal computer (Delfgauw, The Netherlands), equipped with a Chromeleon chromatographic data system (Gynkotek HPLC). The chromatographic system could be extended with a Spectroflow 757 absorbance detector (Kratos Analytical, Westwood, NJ, USA)

Mass spectrometry (MS) was performed using flow injection analysis with a LC-9A pump (Shimadzu, Kyoto, Japan) on a VG Platform II mass spectrometer (MicroMass, Altrinchem, UK).

2.3. MS conditions

The sample (10 μl) was introduced in the electrospray interface in the positive ion mode with a 10 μl/min flow-rate of 0.1% (v/v) formic acid in acetonitrile–water (50:50, v/v). The total mass range, 100–600, was scanned in 7.1 s per cycle. The mass resolution was 13 (instrumental units), the cone voltage 30 V and the source temperature 80°C.

2.4. Chromatographic conditions

Partial-loop injections (20 μl) were made on a Chromspher C_8 column (150×4.6 mm, d_p=5 μm, average pore diameter=13 nm, Chrompack, Middelburg, The Netherlands) with a R3 pre-column (10×3 mm, Chrompack). The column temperature was maintained at 40±2°C. The eluent comprised 10% (v/v) acetonitrile and 90% (v/v) of a buffer (pH 7.0) containing 10 m*M* sodium phosphate and 2 m*M* tetrabutylammonium-hydrogen sulfate; the eluent flow-rate was 1.5 ml/min. The fluorescence detection wavelengths were 254 nm for excitation and 425 nm for emission, using a 16-nm slit width for emission.

2.5. Analytical procedures

To a 200-μl plasma sample, pipetted into a polypropylene micro tube (1.5 ml, Sarstedt, Nümbrecht, Germany), 50 μl of 1 μg/ml PMPA, tested as an internal standard, in water was added; the sample was then vortex-mixed. The proteins were precipitated adding 50 μl of 20% (w/v) TCA in water followed by extensive vortex-mixing. After centrifugation at $1.3 \cdot 10^3$ *g* for 15 min, 150 μl of the supernatant was pipetted into another tube and 50 μl of a mixture of 2 *M* sodium acetate and 160 m*M* chloroacetaldehyde in water was added. After vortex-mixing, the tightly closed micro tube was heated at 98°C for 30 min in a thermostated water bath. After cooling down the tube in the refrigerator at 2°C and vortex-mixing of the sample the clear yellowish reaction mixture was transferred into a glass insert in an injection vial.

For the MS identification of the phosphonic acids and their derivatives, solutions of approximately 1 mg of each compound were made in 1 ml of a mixture of 0.5 *M* sodium acetate and 40 m*M* chloroacetaldehyde in a polypropylene micro tube. Half of both samples was subjected to the standard reaction conditions, the other half was kept as a reference solution. Prior to injection, the samples were diluted 100-fold with 0.1% (v/v) formic acid in acetonitrile–water (50:50, v/v).

2.6. Validation

Two stock solutions of approximately 50 μg/ml adefovir in 10 m*M* phosphate buffer (pH 6.0) were prepared by separate weighing and were stored at −20°C. A stock solution of 50 μg/ml PMPA in 10 m*M* phosphate buffer (pH 6.0) was made and stored at 2°C. For calibration, a 1 μg/ml calibration sample in drug-free pooled plasma, originating from four individuals, was made from one of the 50 μg/ml adefovir stock solutions and stored at −20°C. Dilutions of this calibration sample yielded 10, 25, 50, 100, 250 and 500 ng/ml adefovir calibration samples in pooled plasma and were made daily for each analytical run. Each sample was prepared in duplicate for the calibration. For PMPA a dilution of 1 μg/ml in water was prepared daily. Least-squares linear regression, weighted by $x^{-1.5}$ (reciprocal of the concentration∧1.5) was employed for the calibration, using the peak height of 1,N^6-etheno-adefovir. The power of the weighing factor was determined by maximizing the log-likelihood function and the linearity of the calibration was tested using a lack-of-fit test; both statistical procedures were performed using the SPSS 7.5 software (SPSS, Chicago, IL, USA).

From the other 50 μg/ml stock solution of adefovir validation samples plasma were prepared at 10, 40, 200 and 1000 ng/ml, respectively and stored at −20°C; plasma of different individual donors was used for each sample. Precisions and accuracies were determined by six-fold analysis of each validation sample in three different analytical runs, including the sample pre-treatment. The repeatability (intra-assay precision) is calculated according to

$$\text{Repeatability} = \frac{\sqrt{\text{ErrMS}}}{\text{GM}} \times 100\% \quad (1)$$

where ErrMS = error mean square, GM = grand mean and the reproducibility (inter-assay precision) according to

$$\text{Reproducibility} = \frac{\sqrt{(\text{DayMS} - \text{ErrMS})/n}}{\text{GM}} \times 100\% \quad (2)$$

where DayMS = day mean square, n = number of replicates in each run) for each individual concentration.

Six individual blank plasma samples were processed to test the selectivity of the assay. The selectivity of the assay was also tested by investigating the influence of several pharmaceutical compounds, including a few of their metabolites, potentially used by patients treated with adefovir. Dilutions of 5 or 10 μg/ml of all 26 tested agents were made in drug-free plasma (five or six different drugs per sample) and processed in duplicate according to the standard procedure.

In addition, the stability of adefovir in plasma was tested at the 40, 200 and 1000 ng/ml concentration level at −20°C, ambient temperature, 37°C and 60°C, respectively, for different time intervals, relevant at each temperature. In addition, the effect of four additional freeze–thaw cycles was investigated.

For the determination of the extraction yield, calibration samples of adefovir in (with TCA) deproteinized plasma made from the same stock as the calibration samples in plasma, in the 10–1000 ng/ml range is used. During pre-treatment of these samples water was added as a replacement of the TCA solution. The yield (Y) was calculated for two calibrations in two separate analytical runs by dividing the slopes of the two different calibration lines and by correcting for the normal plasma protein content (75 mg/ml): $Y = (B_n/B_{ref})(V_s - V_p)/V_s = (B_n/B_{ref})(300-15)/300 = 0.95(B_n/B_{ref})$, where B is slope of calibration line; n, the normal calibration samples; ref, the reference calibration samples (without proteins); V_s, the sample volume and V_p, the normal protein volume (200 μl plasma).

The stability of 1,N^6-etheno-adefovir stored in the autosampler at ambient temperature for 14 h was verified. The recovery was calculated analogously to the extraction yield ($Y = B_{ref}/B_n$).

3. Results and discussion

From the existing pre-treatment procedures for adefovir in plasma, protein removal was expected to be necessary; for the simplification of the sample treatment we therefore focussed on the direct combination of protein precipitation and the derivatization with chloroacetaldehyde (Fig. 2). TCA was chosen

as the precipitating agent because of its proven applicability for this goal [7] and the corresponding low dilution factor. Furthermore, the presence of perchlorate, from the alternative precipitating agent perchloric acid, caused the formation of another fluorescent derivative of both adefovir and PMPA with shorter retention times. For the derivatization, the reaction time was varied at 98°C as the reaction temperature (Fig. 3) in order to determine an optimum; 30 min was used for the final assay. The identity of adefovir and their derivatives was confirmed using electrospray-MS (Fig. 4). Analysis of the MS samples with the standard chromatographic system extended with the absorbance detector at 260 nm showed a conversion of ca. 72% for adefovir and ca. 73% for PMPA. Due to interference of reaction products of the derivatization, the yield of the derivatization reaction could not be determined in the concentration range of the analytical assay.

Examples of chromatograms at different concentrations of adefovir spiked to plasma are shown in Fig. 5. Using PMPA as an internal standard (I.S.) a significant lack-of-fit ($P>0.95$) was observed in five out of six calibration lines, while this was only observed in 1 out of 6 calibration lines when the I.S. was ignored. A more close examination of the PMPA peak heights showed a slight increase of the response as a function of the adefovir concentration (ca. 2–10% in the six different runs in the 10–1000 ng/ml concentration range of adefovir), probably causing the non-linearity of the calibration lines if the I.S. was included. The increase of the PMPA response was not caused by contamination of adefovir with PMPA. Peak area were not applied for quantification because of the difficult accurate integration of small 1,N^6-etheno-adefovir peaks due to small, not totally resolved, interfering peaks.

The results obtained with the validation samples (precisions and accuracies at each level in three different analytical runs) are listed in Table 1. The lowest level, 10 ng/ml, proves to be the LLQ. All values of the precision and the accuracy do far meet the demands for a bioanalytical assay: $\leq$20% for the LLQ and $\leq$15% at higher concentrations [9]. Typically, the validation results do also meet these demands if the I.S. is used, the accuracy even is slightly improved then (Table 1), despite the lack of linearity of the calibration lines.

In six individual blank plasma samples, no interferences in the chromatograms are observed which could influence the quantification of adefovir in the validated concentration range. In addition, no co-elution of extra peaks with 1,N^6-etheno-adefovir was observed if 5 or 10 μg/ml of, respectively, 3′-amino-3′-deoxythymedine delavirdine, didanosine, efavirenz, fluconazole, folic acid, ganciclovir, indinavir, itraconazole, lamivudine, methadone, nelfinavir, nevirapine, oxazepam, pyrimethamine, pyrazinamide, ranitidine, rifampin, ritonavir, saquinavir, stavudine, sulfamethoxazole, trimetho-

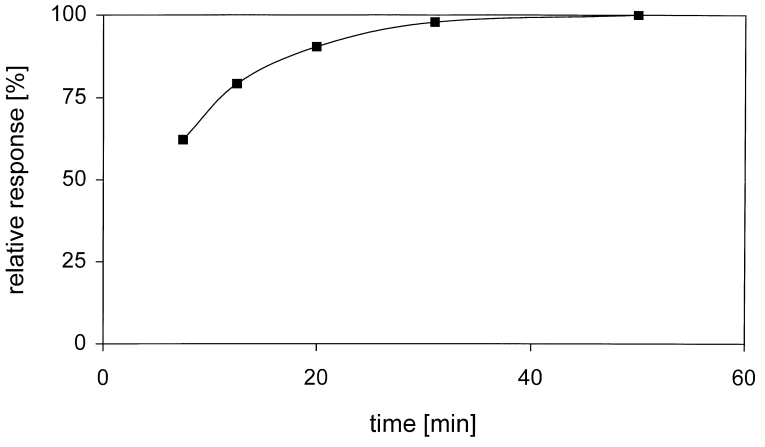

Fig. 3. Fluorescence of 1,N^6-etheno-adefovir as a function of the reaction time. Samples from a standard aqueous solution containing 2.5 μg/ml adefovir were subjected to the standard derivatization procedure using different reaction times.

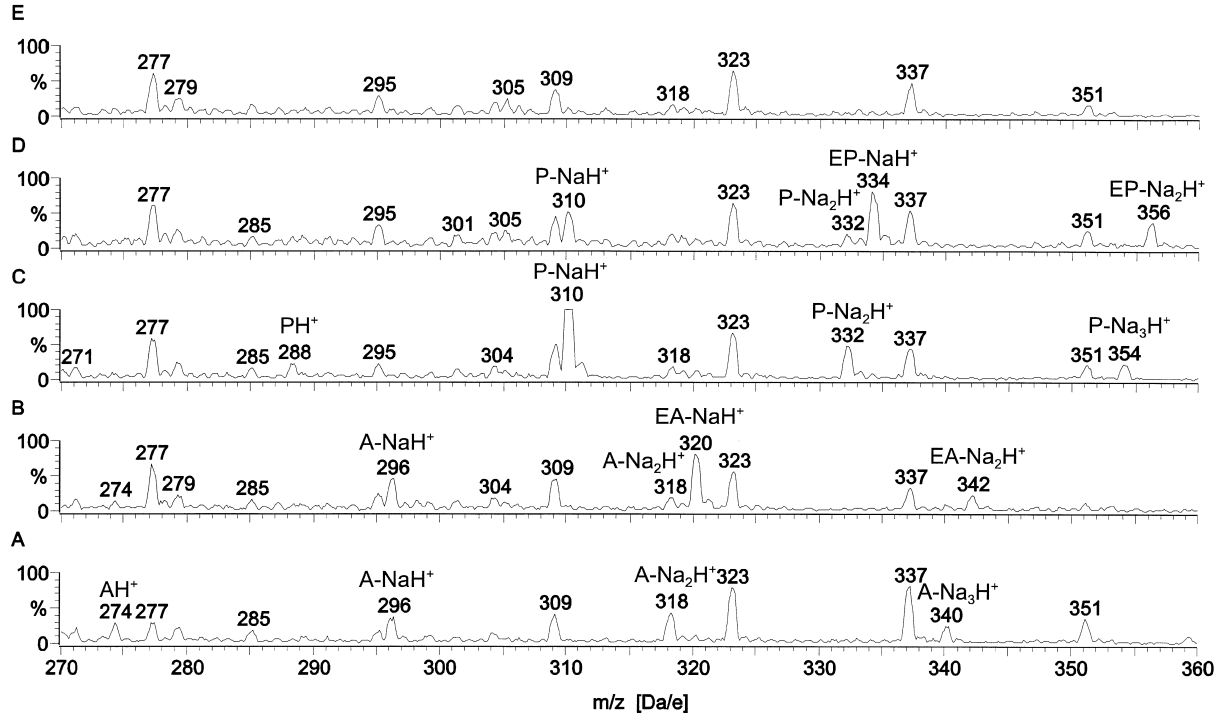

Fig. 4. +Electrospray-MS spectra of (A) adefovir, (B) adefovir after derivatization, (C) PMPA, (D) PMPA after derivatization and (E) blank derivatization. E: 1,N^6-Etheno; A: adefovir; P: PMPA.

prim, zalcitabine, zidovudine (AZT) and AZT-glucuronide is present in the processed plasma sample.

The stability of adefovir in the plasma samples in the present therapeutic relevant concentration range has been investigated; adefovir showed to be stable under all conditions tested, including undergoing four additional freeze–thaw cycles (Table 2); long term storage at −20°C is possible for at least 4.5 months. Unfortunately, storage of adefovir in plasma under non-frozen conditions decreases the precision of the assay at the lowest level tested (40 ng/ml), the investigated storage times at these conditions should thus not be exceeded.

The recovery of adefovir after protein precipitation

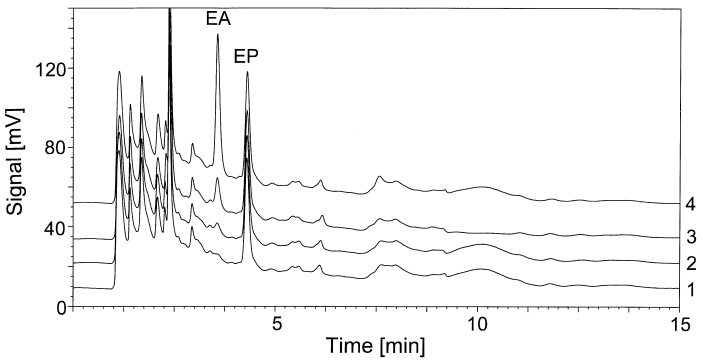

Fig. 5. Chromatograms of 1,N^6-etheno-adefovir in plasma. (1) Blank; (2) 10 ng/ml spiked blank plasma; (3) 50 ng/ml spiked blank plasma; (4) 250 ng/ml spiked blank plasma. EA: 1,N^6-Etheno-adefovir; EP: 1,N^6-etheno-PMPA.

Table 1
Overall results of the validation samples ($n=18$) if the assay is used with and without[a] I.S.

c (ng/ml)	Repeatability (%)		Reproducibility (%)		Accuracy (%)	
10	14	*13*	2	*3*	106	*117*
40	5	*5*	1	*0*	99	*102*
200	3	*3*	2	*0*	101	*95*
1000	2	*2*	2	*1*	95	*95*

[a] Italics indicate the validation parameters for calibration without using the I.S.

Table 2
Recovery of adefovir in plasma ($n=3$) after storage under different conditions using calibration without I.S.

Temperature (°C)	Storage time	Recovery (%)		
		40 ng/ml	200 ng/ml	1000 ng/ml
60	2 h	100±15	95±1	98±1
37	4 h	111±11	99±1	97±1
Ambient	3 days	105±17	96±0	97±2
−20[a]	4 days	95±10	97±4	98±2
−20	4.5 months	107±4	99±2	102±2[b]

[a] The samples were also subjected to four additional freeze–thaw cycles.
[b] $n=5$.

is 101% and is reproducible. The recovery of 1,N^6-etheno-adefovir after storage in the autosampler for 14 h at ambient temperature is 100%.

4. Conclusions

In the presented, simplified assay for adefovir in plasma unnecessary sample treatment procedures [3,4,7] have been avoided, making the assay less labor-intensive; gradient elution and the use of an I.S. are also redundant. Furthermore, the assay is 20–25-fold more sensitive than the earlier reported methods, the precision has been strongly improved [4,7] and the structure of 1,N^6-etheno-adefovir has been confirmed.

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