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Liquid chromatographic assay for the antiviral nucleotide analogue tenofovir in plasma using derivatization with chloroacetaldehyde

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Abstract

A sensitive and selective reversed-phase liquid chromatographic assay for tenofovir in human plasma has been developed and validated. Tenofovir was isolated from a 200 μl plasma sample using protein precipitation with trichloroacetic acid. The fluorescent 1,N^6-etheno derivative is formed at 98 °C in the buffered extract with chloroacetaldehyde. This derivative was analysed using gradient ion-pair liquid chromatography and fluorescence detection at 254 nm for excitation and 425 nm for emission. In the evaluated concentration range (20–1000 ng/ml), the intra-day precision was 4% and the inter-day precision was 5–6%. An accuracy of between 97 and 110% was determined. The lower limit of quantification was 20 ng/ml with an inter-day precision of 11%, an intra-day precision of 12% and an accuracy of 103%. The assay is subject to interference from co-administered abacavir. The usefullness of the assay was demonstrated for samples obtained from an HIV-infected patient treated with tenofovir.

Keywords: Derivatization, LC; Tenofovir; Chloroacetaldehyde

1. Introduction

Tenofovir {9-[(*R*)-2-(phosphonomethoxy)propyl]-adenine, PMPA, Fig. 1A}, a nucleotide reverse transcriptase inhibitor, is one of a large number of antiviral agents that play a role in current anti-human immunodeficiency virus (HIV) therapy [1]. For oral use, the prodrug tenofovir disoproxil (Fig. 1B), formulated as the fumarate salt, has been developed [2,3]. After hydrolysis of the prodrug, tenofovir is intracellularly phosphorylated into the active metabolite tenofovir diphosphate (Fig. 1C). So far, no major toxicity has been discovered for this drug [4]. Tenofovir disoproxil fumarate (Viread®) has already completed phase III clinical trials [1] for the treatment of HIV infections with combination therapies of pre-treated patients and has been introduced onto the market. The application of tenofovir in initial therapy is under investigation [4].

Bioanalytical assays provide pivotal data for the pharmacokinetic evaluation of drugs in clinical

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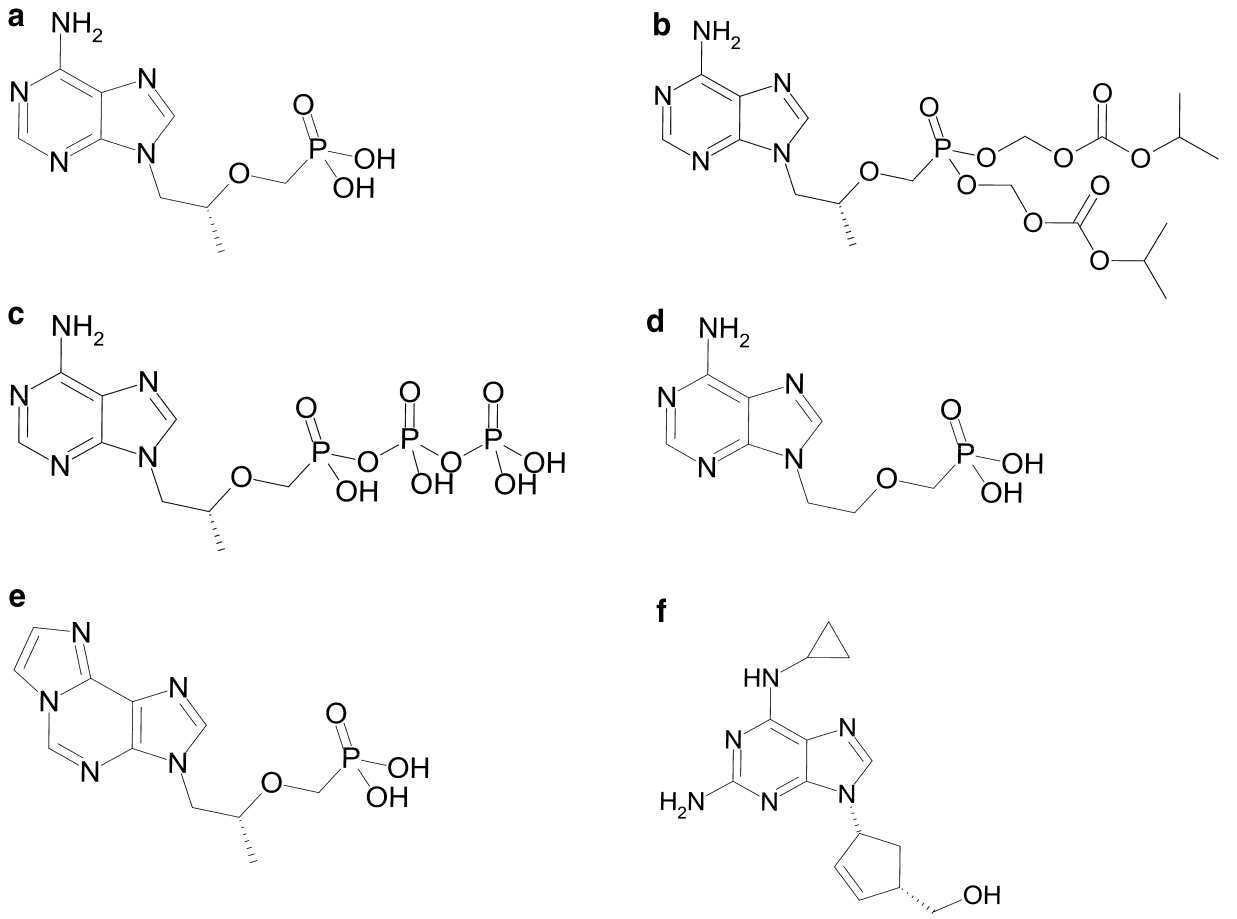

Fig. 1. Chemical structures of (A) tenofovir, (B) tenofovir disoproxil, (C) tenofovir diphosphate, (D) adefovir, (E) 1,N^6-etheno-tenofovir and (F) abacavir.

studies. Although an assay for the intracellular levels of the active metabolite is preferred, an assay for tenofovir may also be valuable. For tenofovir [3,5–7] and its ethyl analogue adefovir (Fig. 1D) [8–10], the use of derivatization of the drug with chloroacetaldehyde is a well-known procedure because the resulting fluorescent 1,N^6-etheno derivatives of these adenine analogues (Fig. 1E) can be sensitively detected using liquid chromatographic methods [9]. In addition, an LC–MS assay has been reported for tenofovir, tenofovir diphosphate, two new prodrugs of tenofovir, GS-7097 and GS-7171, and the metabolite GS-7161 in dog mononuclear cells or in in vitro test cells [11]. The most simple assay using derivatization with chloroacetaldehyde was developed previously in our laboratories for adefovir [10] and comprised protein precipitation, centrifugation, derivatization and chromatographic analysis. No validated assay for tenofovir using derivatization with chloroacetaldehyde has been reported hitherto. A validated chromatographic assay for tenofovir has been reported based on the assay previously developed for adefovir [10]. Essential new selectivity data have been included and the usefullness of the method is demonstrated by the analysis of samples from an HIV-infected patient.

2. Experimental

2.1. Chemicals

Tenofovir monohydrate (referred to below as tenofovir) and adefovir were obtained from Gilead Sciences (Foster City, CA, USA). The pharmaceutical agents used for testing for potential analytical interferences were obtained as pharmaceutical grade reference materials. Blank, drug-free human plasma, containing citrate, phosphate and dextrose as anticoagulants, was obtained from the Saquin Bloedbank (Utrecht, Netherlands). Acetonitrile (gradient grade) was provided by Biosolve (Valkenswaard, Netherlands) and chloroacetaldehyde (50%, w/w) in water was from Aldrich (Milwaukee, WI, USA). Trichloroacetic acid (TCA, reagent grade) was obtained from Riedel-de Haën (Sigma–Aldrich, Seelze, Germany) and tetrabutylammonium hydrogensulphate (ca. 98%) was from Aldrich (Steinhein, Germany). Water was purified by reversed osmosis on a multi-laboratory scale and all other chemicals were of analytical grade from Merck (Darmstadt, Germany)

2.2. Equipment

Chromatographic analyses were performed using the following configuration: a Model 616 pump with column thermostat (Waters Chromatography, Milford, MA, USA), a Waters in-line degasser, a Waters 717plus autosampler and an RF-10Ax1 fluorescence detector (Shimadzu, Kyoto, Japan). Data were recorded on a Model 300GL personal computer (IBM, New York, NY, USA), equipped with a Class-VP chromatography data system (version 4.2, Shimadzu). Also, a Model 5417R centrifuge (Eppendorf, Hamburg, Germany) and a water bath with a Medingen E1 thermostatic heater (Dépex, de Bilt, Netherlands) were used.

2.3. Chromatographic conditions

Partial-loop injections (50 μl) were made on a Chromspher C_8 column (150×4.6 mm, $d_p = 5$ μm, average pore diameter 13 nm, Chrompack, Middelburg, Netherlands) with an R3 pre-column (10×3 mm, Chrompack). The column temperature was maintained at 40 °C. Eluent A comprised a buffer (pH 7.0) containing 10 m*M* sodium phosphate and 5 m*M* tetrabutylammonium hydrogensulphate and eluent B comprised a mixture of this buffer with 50% (v/v) acetonitrile. The eluent flow-rate was 1.5 ml/min. The gradient run started with 5% B in the eluent and was increased to 100% B over 14 min after injection using the concave gradient curve 8. After maintaining this composition for 2 min the column was conditioned with 5% B for 10 min before the next injection. The fluorescence detection wavelengths were 254 nm for excitation and 425 nm for emission.

2.4. Sample pre-treatment

To a 200 μl plasma sample, pipetted into a polypropylene microtube (1.5 ml, Sarstedt, Nümbrecht, Germany), 50 μl of 1 μg/ml adefovir (internal standard) in water was added; the sample was then vortex-mixed. The proteins were precipitated by adding 50 μl of 20% (w/v) trichloroacetic acid in water followed by thorough vortex mixing. After centrifugation at $15 \cdot 10^3$ *g* for 5 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 closed microtube was heated at 98 °C for 45 min in a thermostatic water bath. After cooling the tube in a refrigerator at 4 °C and vortex mixing of the sample, the clear yellow reaction mixture was transferred into a 250 μl glass insert in an injection vial.

2.5. Validation

Three stock solutions of 42.8 and 61.1 μg/ml tenofovir and 41.1 μg/ml adefovir (internal standard) were prepared in 10 m*M* phosphate buffer (pH 6) and stored at −30 °C. For calibration, dilutions of the 42.8 μg/ml tenofovir stock solution were prepared daily, yielding 22, 45, 112, 224, 448 and 1120 ng/ml tenofovir calibration samples in pooled human plasma, respectively, and analysed in duplicate for each analytical run. Least-squares linear regression, using the individual samples and weighted by X^{-2} (reciprocal of the squared concentration), was em-

ployed for the calibration of tenofovir using the ratio (tenofovir/internal standard) of the heights of the peaks of both derivatives.

The 61.1 μg/ml tenofovir stock solution was used to obtain validation (quality control, QC) samples in human plasma at 19.5, 55.0, 244 and 917 ng/ml tenofovir, respectively. These four samples are called QC-LLQ, QC-low, QC-medium and QC-high, respectively. Plasma from different individual donors was used. The precision and accuracy were determined by six-fold analysis of each validation sample in three analytical runs on separate days for all plasma concentrations. Relative standard deviations were calculated for both the intra-day precision (repeatability) and the inter-day precision (reproducibility).

Six individual plasma samples from different individuals were processed to test the selectivity of the assay as blank, double blank (no internal standard) and LLQ (spiked to yield 19.5 ng/ml tenofovir) samples, respectively. The selectivity of the assay was further tested by investigating the chromatographic response in the presence of potentially co-administered drugs. Reference solutions of these drugs were diluted with plasma containing 19.5 ng/ml tenofovir to obtain samples with morphine (70 μg/ml), acetaminophen (22 μg/ml), caffeine (22 μg/ml), lopinavir (10 μg/ml), amprenavir (5 μg/ml) and abacavir (Fig. 1F, 2 μg/ml), respectively.

For the determination of the extraction yield of tenofovir, three reference samples were prepared in (with TCA) deproteinized plasma at the concentrations of the validation samples QC-low, QC-medium and QC-high. All these reference samples were processed and measured six-fold in each of three runs together with the QC samples. The recovery of the extraction of tenofovir was calculated using the ratio of the response of 1,N^6-etheno-tenofovir from the QC samples and the deproteinized plasma reference samples. The values obtained were multiplied by 0.95 as correction for the normal plasma protein content (75 mg/ml) of the QC samples [10].

The stability of tenofovir was investigated for the QC-low and QC-high samples in quadruplicate after exposure to the following conditions: three extra freeze–thaw cycles, 24- and 42-h exposure of the plasma samples to ambient temperature, 1-h exposure of the biological samples at 60 °C in a water bath (leading to inactivation of HIV) [12], 1 week and 2 months storage of QC samples at 4 °C and 22 h storage of the final samples in the autosampler at ambient temperature.

An HIV-infected patient received 300 mg tenofovir disoproxil fumarate orally (daily, at steady state) in addition to zidovudine, lamivudine, didanosine, allopurinol, granisetron and doxorubicin. Blood samples were taken at nine different time points over 7 h for pharmacokinetic evaluation. Heparin tubes were used and plasma was separated by centrifugation for 10 min at ~1500 *g* and the samples were analysed once using the reported assay.

3. Results and discussion

The validated chromatographic assay for tenofovir was based on a previously validated assay for adefovir [10]. The derivatization time, however, was increased to 45 min (Fig. 2) for a maximal yield of 1,N^6-etheno-tenofovir and 1,N^6-etheno-adefovir. In our opinion, the minor 1–2% increase in the yield during the last quarter of the reaction time indicates that further prolongation of the reaction will not be significant. The structure of these derivatives has been confirmed previously using positive electro-

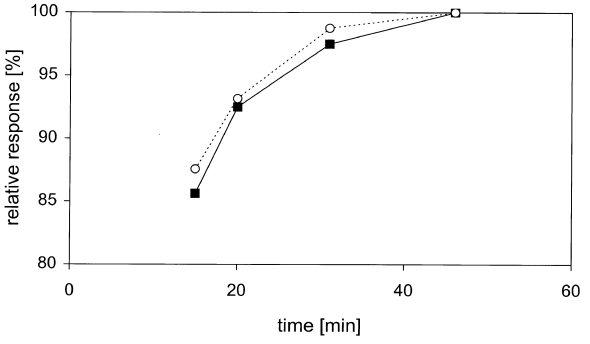

Fig. 2. Relative yield of 1,N^6-etheno derivatives as a function of reaction time during derivatization of tenofovir (■) and adefovir (○) with chloroacetaldehyde. Samples from a standard aqueous solution containing 214 ng/ml tenofovir and 206 ng/ml adefovir were subjected to the standard derivatization procedure for different reaction times ($n=3$).

spray mass spectrometry [10]. For the tenofovir (PMPA) derivative the monosodium and disodium parent ions were observed, as expected, at 334 and 356 Da/e, respectively. For optimal selectivity, the concentration of the ion-pairing agent was increased and gradient elution was used. The chromatograms are shown in Fig. 3.

3.1. Validation

The assay was linear over the concentration range 21–1120 ng/ml in human plasma, as is shown in Table 1. For six calibration curves the calibration concentrations were back-calculated from the ratio of the peak heights. The deviation of the nominal concentration was ≤2% (Table 1) for all concentrations without any sign of non-linearity. The weighted linear regression parameters for these curves (±SD, $n=6$) were: intercept 0.010±0.007, slope 4.16±0.39 ml/μg and standard error 0.008±0.002.

Assay performance data are presented in Table 2. For the lowest QC sample (20 μg/ml) the accuracy and precision were within the ±20% range; this value can therefore be used as the lower limit of quantification (LLQ). The accuracies and precisions for the other tested concentrations were all within the required ±15% [13–15]. The LLQ is analogous to previously reported values using the same analytical approach [5–7] or LC–MS [11].

The analysis of six batches of control plasma showed no endogenous constituents co-eluting with either tenofovir or the internal standard. All tested potential co-medications and metabolites showed no

Table 1
Back-calculated concentrations ($n=6$) of tenofovir in plasma calibration samples

Nominal conc. (ng/ml)	Conc. found ±SD (ng/ml)	Reproducibility (%)	Accuracy (%)
22.4	22.6±1.2	5.1	101
44.8	43.9±1.9	4.4	98
112	112±4	3.4	100
224	226±7	3.0	101
448	448±13	2.9	100
1120	1120±48	4.3	100

Table 2
Assay performance data for the determination of tenofovir ($n=18$)

Nominal conc. (ng/ml)	Repeatability (%)	Reproducibility (%)	Accuracy (%)
19.5	11	12	103
55.0	3.9	6.1	110
244	4.2	5.1	97
917	4.1	5.3	110

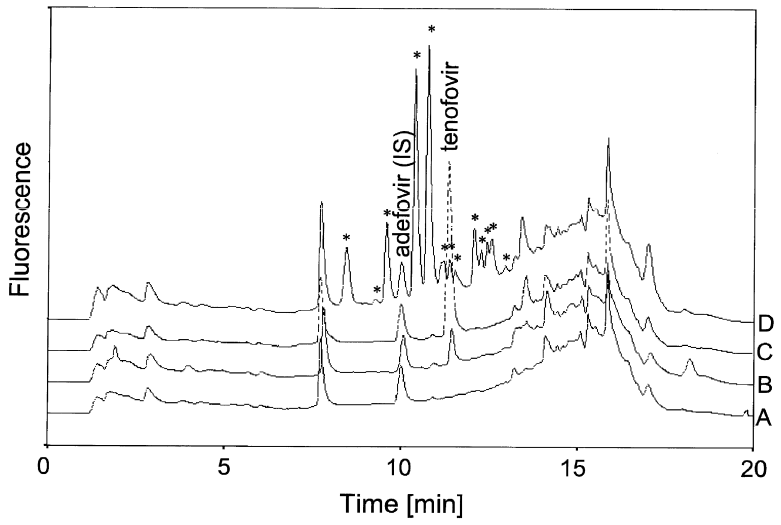

Fig. 3. Chromatograms of (A) blank human plasma with the internal standard adefovir added, (B) a patient sample containing 197 ng/ml tenofovir, (C) a spiked plasma sample containing 1120 ng/ml tenofovir and (D) a spiked plasma sample containing 2 μg/ml abacavir. (*) Abacavir-related peaks.

Table 3
Stability data for tenofovir [recovery±SD (%) ($n=4$)]

Conditions	55 ng/ml	919 ng/ml
Three extra freeze–thaw cycles (plasma)	96.6±2.4	102.2±5.3
24 h ambient (plasma)	95.6±2.6	99.9±2.1
42 h ambient (plasma)	103.2±11.8	96.5±6.3[a]
1 h 60 °C (plasma)	98.4±7.9	94.1±0.1[a]
1 week 4 °C (plasma)	92.3±2.9	100.2±4.5
2 months 4 °C (plasma)	104.4±3.8	98.8±2.8
22 h ambient (final samples)	101.8±1.0	102.1±1.9

[a] $n=3$.

interference, except for the purine derivative abacavir (Fig. 3). The interference of this nucleoside reverse transcriptase inhibitor can be explained by its structural similarity to tenofovir (Fig. 1). Apparently, abacavir forms several 1,N^6-etheno derivatives with chloroacetaldehyde. An explanation for the formation of multiple fluorescent abacavir derivatives may be the reaction of chloroacetaldehyde with the remaining amine functions of 1,N^6-etheno-abacavir, giving di- or polymeric reaction products. Twenty-six other relevant pharmaceutical agents and a few of their metabolites have previously been tested successfully for their potential interference [10], with none of these agents producing additional peaks in the chromatogram. The high selectivity is due to the selective derivatization reaction in combination with selective fluorescence detection.

The average recovery (±SD) obtained for tenofovir was 83.7±2.1% at 917 ng/ml, 82.7±2.2% at 244 ng/ml and 88.1±3.8% at 55 ng/ml. Stability data for the analytes in plasma and the final sample are presented in Table 3. No degradation was observed under all conditions tested, including 2 months storage of plasma samples at 4 °C.

Finally, the pharmacokinetic curve for tenofovir in an HIV-infected patient is demonstrated in Fig. 4 and shows that the assay can be applied for the analysis of plasma from HIV-infected individuals treated with tenofovir.

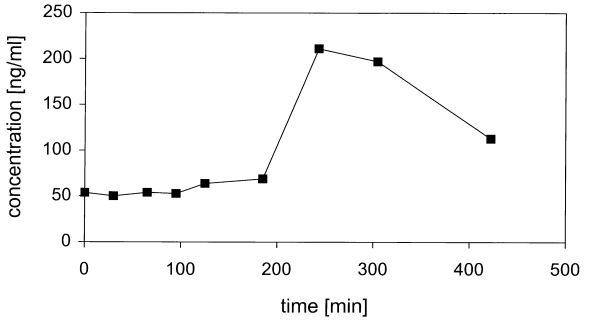

Fig. 4. Pharmacokinetic curve of tenofovir in plasma from a patient receiving 300 mg tenofovir disoproxyl fumarate daily.

4. Conclusion

A selective and sensitive HPLC assay meeting common validation criteria was developed for the quantification of tenofovir. The compound is stable under all conditions tested. The assay can be used to analyse plasma from HIV-infected patients treated with the drug. It is advised not to use this assay if abacavir is co-administered.

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