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Short communication

Determination of tenofovir in human plasma by high-performance liquid chromatography with spectrofluorimetric detection

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Abstract

A sensitive high-performance liquid chromatography method was developed with spectrofluorimetric detection for the determination of tenofovir, a new HIV reverse transcriptase inhibitor, in human plasma. After precipitation of 200 μl of plasma samples by methanol and evaporation of the supernatant, fluorescent derivatized compounds were obtained by a 40-min incubation at 80 °C with chloroacetaldehyde 0.34% at pH 4.5. The assay was performed isocratically using 5 m*M* Na_2HPO_4 (pH 6), containing tetrabutylammonium (TBA) chloride 5 m*M*, and acetonitrile (85:15, v/v) as mobile phase, and a Cluzeau C_8 plus satisfaction column maintained at 35 °C. Detection was performed at excitation and emission wavelengths set at 236 and 420 nm, respectively. In these conditions, tenofovir can be separated from adefovir, the internal standard, and endogenous substances. The method was found to be linear and has been validated over a concentration range of 5–1000 μg/l. The average coefficient of the limit of quantification (5 μg/l) was 5.38% and at this concentration, a signal-to-noise ratio of 500 was measured

Keywords: Tenofovir

1. Introduction

Tenofovir (or 9-[(*R*)-2-(phosphonomethoxy)-propyl]adenine) is a new nucleotide antiretroviral drug used in human immunodeficiency virus type 1 (HIV-1) infection. To improve its low bioavailability, a prodrug of tenofovir, tenofovir disoproxil fumarate, is used. The drug has an elimination half-life of 15 h [1] and is metabolised intracellularly to tenofovir diphosphate which is an inhibitor of HIV-1 reverse transcriptase. A potential asset of tenofovir is its ability to be active against a variety of drug-resistant HIV-1 strains in vitro [2,3]. A previous method has been reported for the assay of tenofovir by high-performance liquid chromatography (HPLC) and fluorimetric detection with a limit of quantification of 25 μg/l [4]. The method presented here uses the same detection but provides a smaller limit of quantification (5 μg/l) which should allow the determination of most plasma concentrations.

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2. Experimental

2.1. Chemicals and reagents

Tenofovir hydrate and the internal standard (I.S.) adefovir were kindly supplied by Gilead (Foster City, USA). Lichrosolv® gradient grade acetonitrile for HPLC, anhydrous sodium acetate GR (>99%) and disodium hydrogen phosphate dihydrate GR for analysis (>99.5%) were obtained from Merck (Darmstadt, Germany). Rectapur® orthophosphoric acid 85% and Chromanorm® methanol for HPLC were obtained from Prolabo (Paris, France). Acetic acid (99–100%), tetrabutylammonium chloride hydrate and chloroacetaldehyde 50% were from Labosi (Elancourt, France), Sigma (St. Louis, MO, USA) and Aldrich (Steinheim, Germany), respectively. The derivatization reagent (0.34% chloroacetaldehyde in 100 m*M* acetate buffer, pH 4.5) was prepared just before use.

2.2. Chromatographic conditions

The HPLC system consisted of a Beckman Coulter 114M pump (Villepinte, France), a WATERS WISP 710P autosampler (Millipore, Saint-Quentin-en-Yvelines, France), a CROCO-CIL column oven (Beckman Coulter). The chromatographic separation was accomplished on a C_8 plus satisfaction column (250×3 mm, 5 μm; Cluzeau, Sainte Foy la Grande, France) protected by a guard column (15×3 mm) and a A-103X filter (Cluzeau). The column was maintained at 35 °C. The standard mobile phase consisted of phosphate buffer (5 m*M*, pH 6) containing 5 m*M* tetrabutylammonium chloride–acetonitrile (85:15, v/v) and was filtered through a 0.45 μm filter before use. The flow rate was 0.5 ml/min. The fluorescence of the derivatized compounds was monitored with excitation and emission wavelengths set at 236 and 420 nm, respectively, using a fluorimetric detector RF-551 (Shimadzu, Croissy Beaubourg, France).

2.3. Standard preparation

Two independent stock solutions of tenofovir 1000 μg/ml were made by dissolving tenofovir hydrate equivalent to 10 mg anhydrous tenofovir in 10 ml of water. A stock solution of internal standard was made by dissolving 10 mg of adefovir in 10 ml of water. All these solutions were stored at 4 °C before use. Quality control (QC) samples were prepared at final tenofovir concentrations of 30, 200 and 750 μg/l by spiking drug-free human plasma with appropriate amounts of diluted stock solution in water. After mixing, the QC samples were then stored in propylene tubes at −20 °C. In this storage condition, plasma samples were stable for at least 3 months. Samples (20 μl) of appropriate dilutions of the tenofovir stock solution in water were used to spike 180 μl of blank plasma in order to provide calibration standards over the concentration range 5–1000 μg/l.

2.4. Sample preparation

In hemolysis tubes, 20 μl of a working solution of adefovir (2.5 μg/ml) were added to 200 μl of plasma samples, quality controls and calibration standards. After mixing, samples were deproteinated by the addition of 600 μl of methanol. Samples were then vortex-mixed for 30 s and centrifuged for 10 min at 2200 *g*. The supernatant was transferred to a screw-cap tube and evaporated to dryness at 60 °C under a stream of nitrogen, and the dry residue was reconstituted in 200 μl of the derivatization reagent. After adding the screw-cap, the tubes were incubated at 80 °C for 40 min. Derivatized samples were then cooled at −20 °C for 10 min and centrifuged at 2200 *g* for 10 min. The supernatant was transferred from each tube to autosampler vials and 80 μl were injected in the chromatographic system, except for the highest concentration (500, 750 and 1000 μg/l) for which the injected volume was 40 μl.

2.5. Validation procedures

Complete standard curves (5, 10, 25, 50, 100, 250, 500 and 1000 μg/l) were analysed in triplicate on 3 separate days. Quality controls were assayed twice with each standard curve. A linear regression with a weighting factor of $1/(\text{peak height ratio})^2$ was used to plot the peak height ratio of tenofovir to internal standard versus tenofovir concentration. Slope, *y*-intercept and correlation coefficient were calculated for each standard curve. Accuracy, within and be-

tween-day precision of the method were evaluated on the quality controls by multiple analysis, on the pool of 1 assay day and on all the analytical runs, respectively. Accuracy was determined as the difference between back calculated (C_{obs}) and theoretical concentration (C_{theo}) expressed in percent. The limit of quantification was set at the lowest calibration standard value (5 μg/l). The recovery of tenofovir was quantified at concentration levels corresponding to the limit of quantification, and the medium and high calibration standard values (5, 100, 1000 μg/l) analysed in triplicate. The peak height ratio of extracted samples containing both tenofovir and internal standard were compared to the ratio of samples containing the same amount of I.S. but no tenofovir which was added just before the derivatization step. The same method was employed to study the recovery of the I.S. with tenofovir used as reference compound. Freeze–thaw stability of tenofovir was determined by assaying quality control samples in triplicate over three freeze–thawing cycles. Specificity of the method was studied by injecting in the chromatographic system aqueous solutions of derivatized nucleoside analogues.

3. Results

The nine standard curves were linear over a concentration range of 5–1000 μg/l, with a mean slope (±SD) of 3.05±0.30 and a mean *y*-intercept (±SD) of 0.00376±0.00359. The average correlation coefficient was 0.997. The average coefficient of variation for average results of back calculated calibration standard concentrations was 6.68% and the average coefficient of variation for the lower concentration (5 μg/l) was 5.38%. The within-day precision, expressed by the coefficient of variation of observed concentrations was less than 5.7%, while the within-day accuracy, expressed by the calculated bias between observed and theoretical concentrations, ranged from −4.27 to −9.1%. The between-day precision and accuracy were, respectively, less than 9.60 and 11.4% (Table 1). A signal-to-noise ratio of ~500 was measured at the limit of quantification. The mean recovery of tenofovir was 78.8% for the 5 μg/l samples with a coefficient of variation of 4.22%. The mean recovery of the 100 μg/l samples was 79.7% with a coefficient of variation of 1.26% and the 1000 μg/l samples had a mean recovery of 79.6% with a coefficient of variation of 3.55%. The mean recovery of the internal standard was 80% with a coefficient of variation of 2.31%. Freeze–thaw stability of tenofovir was evaluated by the calculated bias between observed and theoretical concentrations (Table 2). These bias ranged from +11.3 to −5% for low control, from +12.8 to −0.5% for the medium control and from +14.1 to −4.67% for the high control. No interference has been found with endogenous substances or with the nucleoside analogues tested (zidovudine, lamivudine, stavudine, abacavir and didanosine). Fig. 1 gives representative chromatograms of blank (A) or plasma spiked (B) with 250 μg/l of tenofovir and 250 μg/l of adefovir.

Table 1
Precision and accuracy of tenofovir determination in human plasma

	Theoretical (μg/l)	Observed (μg/l)	C.V. (%)	Bias (%)
Within-day	30	32.1	5.70	+4.27
(*n*=6)	200	216	3.10	+8.20
	750	818	4.28	+9.10
Between-day	30	32.6	9.60	+8.70
(*n*=18)	200	223	8.40	+11.4
	750	773	7.90	+3.10

Table 2
Freeze–thaw stability of tenofovir in human plasma (*n*=3)

Theoretical (μg/l)	Observed (μg/l)	C.V. (%)	Bias (%)
Freeze–thaw cycle 1			
30	33.4	4.50	+11.3
200	226	3.40	+12.8
750	856	5.90	+14.1
Freeze–thaw cycle 2			
30	26.6	7.10	−11.2
200	200	6.30	0
750	715	3.15	−4.70
Freeze–thaw cycle 3			
30	28.5	3.40	−5
200	199	2.70	−0.500
750	752	5.80	0.300

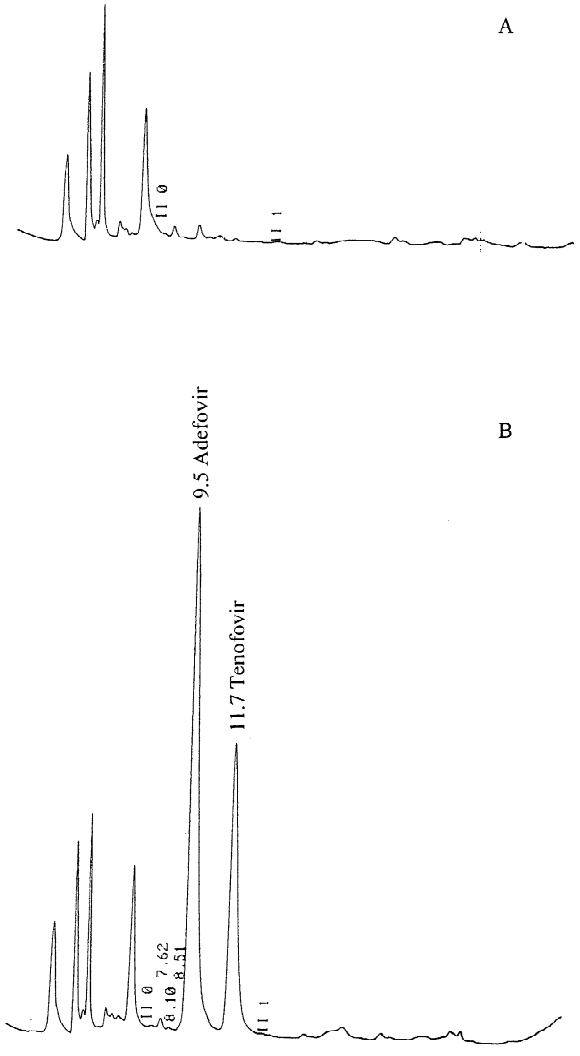

Fig. 1. Chromatograms showing (A) blank plasma and (B) human plasma containing tenofovir (250 μg/l) and adefovir (250 μg/l).

4. Discussion

Tenofovir and adefovir both react with chloroacetaldehyde to form a N^1,N^6-ethenoadenine fluorescent derivative [5]. A previous chromatographic method using this derivatization reaction has been published but the limit of quantification was 25 μg/l, and furthermore no chromatogram was shown. Therefore, a sensitive HPLC assay was developed with a lower limit of quantification. In order to improve the sensitivity of the method a Cluzeau C_8 plus satisfaction column with a smaller diameter (3 mm) was used. The ionic strength of the phosphate buffer was also an important parameter as it must not be above 5 m*M* in order to separate tenofovir from an interfering peak present in plasma.

Peak height is also dependent on the temperature used to derivatize the samples as it has been shown that derivatization was complete after incubating samples at 90 °C for 40 min [5]. Incubating samples at 80 °C rather than 90 °C permitted quantification of higher serum concentrations without the necessity of dilution to prevent fluorimetric detector saturation. As the derivation reaction is not complete when performed at 80 °C, the possible residual derivation that could occur during the analysis has been investigated by incubating calibration standards (1000, 100 and 10 μg/ml) at 35 °C during 1 h. Tenofovir and adefovir peak heights obtained represented less than 0.5% of the peak heights of equivalent samples derivatized for 40 min at 80 °C. These increases were assumed to be negligible, based on the linearity of the calibration standard curve and the good reproducibility obtained on quality control samples.

The injected volume depends on the analysed sample tenofovir concentration. If this concentration is initially unknown for patient samples, it is however possible to have an idea of the expected concentration range as the time span between drug administration and blood sampling is always known. Practically, 40 μl of the extract should be injected if blood was sampled less than 4 h after administration, and 80 μl should be injected if the time between sampling and drug administration was longer. Furthermore, the final volume present in autosampler vials which is equal to 200 μl allows injection of several volumes of the same extract if necessary. The use of different injection volumes and their effect on column efficiency is balanced by the use of an internal standard as peak height ratio is supposed to be independent of the injected volume.

In these conditions, according to the kinetic profile of the drug, plasma concentration measured at steady state before administration of tenofovir should be quantifiable in most patients. The lower limit (5

μg/l) is easily quantifiable as the corresponding signal-to-noise ratio is equal to 500. An even lower limit could have been reached as the true limit of quantification is the concentration for which a response factor equal to ten times standard deviation of noise under assay conditions is measured. However, it was decided to set this limit at 5 μg/l as this concentration seems to be clinically relevant. This was demonstrated by using this method to determine tenofovir concentration in 100 plasma samples from 50 patients. The concentrations ranged from 13 to 95 μg/l 24 h after administration, and from 200 to 1500 μg/l within the first 4 h following administration. None was below the lower limit.

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