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Quantitative determination of (−)-2′-deoxy-3′-thiacytidine (lamivudine) in human plasma, saliva and cerebrospinal fluid by high-performance liquid chromatography with ultraviolet detection

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Abstract

A high-performance liquid chromatographic method for the quantitative determination of the HIV reverse transcriptase inhibitor lamivudine ((−)-2′-deoxy-3′-thiacytidine, 3TC, Epivir®) in human plasma, saliva and cerebrospinal fluid is described. Lamivudine was extracted from samples using silica extraction columns prior to reversed-phase high-performance liquid chromatography with ultraviolet detection at 270 nm. The method has been validated over the range of 10 (lower limit of quantitation) to 5000 ng/ml using a 0.5-ml sample volume. Between-day and within-day precisions ranged from 3.5 to 9.0%. The assay has been used for the quantitative analysis of lamivudine in plasma and cerebrospinal fluid of HIV-1 infected patients.

Keywords: Lamivudine; (−)-2′-Deoxy-3′-thiacytidine

1. Introduction

Lamivudine (Fig. 1) belongs to the class of dideoxynucleoside reverse transcriptase inhibitors, and is a potent in vitro and in vivo inhibitor of human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS) [1–4]. Furthermore, lamivudine shows activity against hepatitis B virus [5,6]. The pharmacokinetic profile of lamivudine in HIV infected patients has not been investigated thoroughly yet.

A high-performance liquid chromatographic

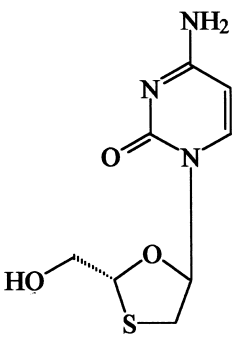

Fig. 1. Molecular structure of lamivudine.

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(HPLC) assay with UV detection for the quantitation of this compound in human urine has been described by Morris et al. [7]. Their assay is linear in the range 0.5–500 μg/ml. An HPLC assay for the determination of lamivudine in human serum has been described by Harker et al. [8]. This assay requires a relatively large volume of sample (1.00 ml); their assay is linear in the range 10–5000 ng/ml. Furthermore, an HPLC methodology for the quantitative determination of lamivudine in perfusion solutions from isolated perfused rat kidney studies was described by Hsyu and Lloyd [9]. Their assay can be exploited in the range 25–10 000 ng/ml.

Recently, a radioimmunoassay for the quantitation of lamivudine in subnanogram per millilitre concentrations was described, which may be of use for the determination of intracellular phosphorylated lamivudine [10]. However, the required radioligand and antiserum are not commercially available.

We report the development and validation of a simple HPLC assay using UV detection for the determination of lamivudine in human plasma, saliva and cerebrospinal fluid in the range 10–5000 ng/ml. Assays for the determination of lamivudine in cerebrospinal fluid and saliva have not been reported hitherto. A sample volume of only 500 μl is required. The assay can be used in a hospital laboratory and has been applied to obtain pharmacokinetic data in HIV infected patients.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Model P1000 solvent delivery pump (Thermo Separating Products, Fremont, CA, USA), a Model AS3000 automatic sample injection device, a Model UV1000 wavelength detector and a Chromjet® integrator (all from Thermo Separating Products). The analytical columns used were a μBondapack phenyl column (300×3.9 mm I.D., 10 μm particle size; Waters, Milford, MA, USA), a Zorbax® SB-C_{18} column (75×4.6 mm I.D., 3.5 μm; Rockland Technologies, Newport, DE, USA), and a Zorbax® SB-CN column (75×4.6 mm I.D., 3.5 μm; Rockland Technologies) protected by a Chromguard® C_{18} column (10×3 mm I.D., Chrompack Nederland, Middelburg, The Netherlands). Analytical runs were processed by the Autolab® Software WINNER 386 System (Spectra Physics). UV spectra of lamivudine solutions in distilled water were recorded with a Model 918 UV–VIS spectrophotometer (GBC Scientific Equipment, Dandenons, Australia).

2.2. Chemicals

Lamivudine (lot GR109714X, AWS535) was a kind gift of Glaxo–Wellcome (Research Triangle Park, NC, USA). Methanol (supra-gradient) and acetonitrile (HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Phosphoric acid 85%, dipotassium hydrogenphosphate and ammonium acetate (all analytical reagent grade) were purchased from Merck (Darmstadt, Germany). Distilled water was used throughout. Blank, drug-free plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, The Netherlands). Blank saliva was provided by healthy volunteers. Blank cerebrospinal fluid was obtained from patients who underwent lumbar puncture for methotrexate monitoring after intrathecal administration of the drug.

2.3. Preparation of standards

Stock solutions of lamivudine were prepared by dissolving the appropriate amount of the drug, accurately weighed, in distilled water to yield a final drug concentration of 0.4 mg/ml. For the construction of calibration curves fresh solutions were used. Drugs for interference analysis were obtained from the hospital pharmacy (Slotervaart Hospital, Amsterdam, The Netherlands), either as solutions for injection or after dissolving solid reference material in 50% (v/v) methanol (final concentration 500 μg/ml).

2.4. Sample pretreatment

For the preparation of the standard samples stock solutions of lamivudine were diluted with distilled

water. To achieve lamivudine calibration concentrations of 10–5000 ng/ml, appropriate quantities of the various diluted solutions were added to blank plasma, saliva or cerebrospinal fluid in Eppendorf tubes (Merck). The solutions were mixed on a vortex mixer for 10 s.

Next, 500 μl of plasma or cerebrospinal fluid was subjected to solid-phase extraction (SPE). To increase recovery from saliva samples, 600 μl of saliva was mixed with 600 μl of blank human plasma. Volumes of 1.00 ml of these mixtures were then, after centrifugation for 3 min at 10 500 *g*, subjected to SPE.

Prior to SPE, silica extraction columns (500 mg, 3 ml; Varian, Harbor City, CA, USA) were placed on a vacuum elution manifold (Baker 12-SPE System; Baker, Phillipsburg, NJ, USA), and rinsed with 2.0 ml of methanol, followed by two aliquots of 2.0 ml of distilled water. The flow-rate was maintained at 1.0 ml/min. Care was taken that the columns did not run dry. Next, 0.5 ml of the plasma or cerebrospinal fluid samples, or 1.0 ml of the diluted saliva samples was transferred onto the columns and drawn into them by applying reduced pressure. The columns were then washed with 0.5 ml of distilled water, followed by vacuum suction for 1 min. Elution of the absorbed analyte into Eppendorf tubes was performed by using two volumes of 0.5 ml of methanol. Methanol was evaporated to dryness under a gentle stream of nitrogen at 40°C. The residues were redissolved in 100 μl of mobile phase, mixed on a vortex mixer for 60 s and centrifuged for 10 min at 10 500 *g*. The clear supernatants were placed in autosampler vials with inserts.

2.5. Chromatography

The chromatographic analysis was performed at ambient temperature on a phenyl analytical column with a mobile phase composed of a 0.005 *M* dipotassium hydrogenphosphate solution in distilled water (pH adjusted to 6.8 with 85% phosphoric acid)–methanol (92:8, v/v). Prior to use, air was purged from the mobile phase with helium. Absorbance was measured at 270 nm. The flow-rate was maintained at 1.0 ml/min. Aliquots of 75 μl were injected.

2.6. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank plasma, saliva and cerebrospinal fluid samples. The following compounds were investigated for interference with the analytical method (including the sample pretreatment): didanosine, fluconazole, folinic acid, ganciclovir, indinavir, methadone, methotrexate, oxazepam, pyrazinamide, ranitidine, rifampin, ritonavir, saquinavir, stavudine, sulfamethoxazole, trimethoprim, zalcitabine, zidovudine and zidovudine glucuronide in a final concentration of 20 μg/ml in plasma.

2.7. Limit of detection and limit of quantitation

The limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. At this concentration a significant difference between the spiked and blank samples is required in plasma from six individuals (two-tailed, paired Student's *t*-test).

The lower limit of quantitation (LLQ) was investigated in plasma samples from six different donors. Each sample was spiked to contain 1, 2 or 3 times the LOD concentration of lamivudine. For the concentration to be accepted as the LLQ, the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (R.S.D., measure of precision) are to be less than 20%. The upper limit of quantitation (ULQ) was arbitrarily defined as 5000 ng/ml.

2.8. Accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precision of the method were determined by assaying two replicate samples of plasma at three different lamivudine concentrations (100, 1500 and 4000 ng/ml) in four analytical runs. Accuracy was measured as the percent deviation from the nominal concentration. The within-day and between-day precision were obtained by analysis of variance (ANOVA) for each concentration using the analytical run as the grouping variable.

Linearity of three calibration curves was tested with the *F*-test for lack of fit, using a weight factor

of [1/conc^2] [11,12]. For the construction of each calibration curve ten spiked plasma samples were analyzed in duplicate.

Recovery of lamivudine after SPE was calculated by comparing the slope of the lamivudine calibration curve in extracted plasma, saliva or cerebrospinal fluid to those of nonprocessed standard solutions.

2.9. Stability

Blank plasma samples were spiked with an aliquot of diluted lamivudine stock solution to give initial concentrations of 100, 1500 and 4000 ng/ml. These samples were stored for 1 h at 60°C, 24 h at 25°C, 7 days at 4°C, 30 days at −30°C, or underwent three freeze–thawing cycles. After the storage period six replicates of these samples were analyzed immediately.

2.10. Analysis of patient samples

Plasma and saliva from HIV-1 infected patients who ingested 150 mg of lamivudine after an overnight fast were assayed for the reverse transcriptase inhibitor. Twelve heparinized blood samples were drawn during a time period of 8 h. Plasma was separated by centrifugation at 3000 *g* for 10 min and was immediately stored at −30°C until analysis. Saliva samples were collected with a Salivette®-collecting device (Sarstedt, Etten-Leur, The Netherlands) by chewing on a cotton roll which contained 20 mg of citric acid to stimulate the saliva flow. Saliva was separated by centrifugation for 10 min at 3000 *g* and was immediately stored at −30°C until analysis.

Cerebrospinal fluid samples were obtained from patients who underwent lumbar puncture for establishing a diagnosis of cerebral toxoplasmosis, and who used lamivudine for at least 4 weeks.

2.11. Statistics

All statistical calculations were performed with the statistical product and service solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated P values were ≤ 0.05.

3. Results and discussion

3.1. Chromatography and detection

Reversed-phase chromatography was initially performed with various mixtures of acetonitrile, methanol, and a 0.1 *M* ammonium acetate solution in distilled water at different pH values with a Zorbax SB-C_{18} column and a Zorbax SB-CN column. However, resolution of lamivudine was either not satisfactory (C_{18} column), or not reproducible over time (CN column). Temperature-controlled chromatography (between 22 and 60°C) did not improve the results.

Resolution of lamivudine greatly improved when a μBondapack phenyl column was used instead. Temperature-controlled chromatography was not required, and a mobile phase consisting of a 0.005 *M* dipotassium hydrogenphosphate solution in distilled water (pH adjusted to 6.8 with 85% phosphoric acid)–methanol (92:8, v/v) showed satisfactory results regarding the reproducibility of the retention time of lamivudine, peak shape, and separation from endogenous compounds.

No suitable internal standard was available. The use of another HIV reverse transcriptase inhibitor as internal standard was not considered since one of these compounds is nearly always coadministered with lamivudine in HIV infected patients in clinical practice. Furthermore, the assay as described gives satisfactory validation results without the use of an internal standard.

Lamivudine demonstrated significant UV absorption. Thus, for assaying lamivudine detection was performed at 270 nm since this was the wavelength of maximal absorption of this compound in the mobile phase.

3.2. Sample pretreatment and recovery

For the development of the currently described method, SPE was initially performed by using cation-exchange cartridges (aromatic sulfonic acid, 500 mg, Bakerbond SPE, J.T. Baker, Philipsburg, NJ, USA), modified after the publication by Harker et al. [8]. However, separation of lamivudine and endogenous compounds was far from optimal. In the literature, two bioanalytical methods for the isolation of

other dideoxynucleoside analogues stavudine (d4T) and zalcitabine (ddC) were described using silica cartridges [13,14]. Though the exact mechanisms at work are not fully understood, the use of silica cartridges greatly improved resolution of lamivudine. The chromatographic run time, however, was increased to 50 min due to the elution of endogenous compounds with a retention time of approximately 42 min. These compounds were not observed when the cation-exchange columns were used.

Recovery of lamivudine after SPE was calculated by comparing the slope of the lamivudine calibration curve in extracted samples to those of nonprocessed standard solutions. When saliva was used, a 0.6-ml volume of blank plasma was added to 0.6 ml of saliva to increase the recovery of lamivudine. Lamivudine did not adsorb to the cotton roll of the Salivette device that was used to collect saliva. The recovery of lamivudine from plasma was 97%, and for cerebrospinal fluid a recovery of 112% was found. Without the addition of blank plasma the recovery from saliva was only 31%. After addition of blank plasma the recovery increased to 72%.

3.3. Specificity and selectivity

Blank plasma, saliva, and cerebrospinal fluid from six different individuals showed no interfering endogenous substances in the analysis of lamivudine (Fig. 2A to C). Potentially coadministered drugs or metabolites tested had retention times that were different from lamivudine (zalcitabine, zidovudine, didanosine, stavudine) or were not detected with the described bioanalytical method.

3.4. Limit of detection and limit of quantitation

The LOD in plasma was 5 ng/ml. At this concentration the signal-to-noise ratio was 3. In addition, the response was significantly different from blank plasma ($P<0.001$). At 10 ng/ml the percent deviation from the nominal concentration and the R.S.D. were both less than 20%. Thus, 10 ng/ml was defined to be the LLQ. At all other concentrations up to the ULQ (5000 ng/ml) the percent deviation from the nominal concentration and the R.S.D. were less than 15%. A typical chromatogram of a plasma sample (527 ng/ml) from a patient who is using lamivudine is shown in Fig. 2D.

3.5. Validation: accuracy, precision, linearity and stability

The results from the validation of the method in human plasma are listed in Table 1. The use of the peak area in combination with a weight factor of [1/conc.^{2}] resulted in a minimal deviation from nominal concentrations. The method proved to be accurate (average accuracy at three concentrations 94–97% of the real concentrations). Within-day precision ranged from 3.5 to 5.6%, and between-day precision ranged from 5.2 to 9.0%. Correlation coefficients (r^2) of calibration curves were >0.99 as determined by least squares analysis.

All calibration curves proved to be linear in the range 10–5000 ng/ml with use of the F-test for lack of fit as an indicator of linearity of the regression model.

The stability of lamivudine at various conditions is shown in Table 2. Under all conditions tested lamivudine is stable with concentrations of at least 91% of the initial concentration.

3.6. Analysis of patient samples

The applicability of the assay for pharmacokinetic research in HIV-1 infected patients was demonstrated. Plasma pharmacokinetics of lamivudine showed large inter-individual variation. The plasma concentration–time profile in a patient after ingestion of 150 mg of lamivudine as determined by the currently described bioanalytical method is shown in Fig. 3.

Analysis of several cerebrospinal fluid samples for lamivudine demonstrated that the drug penetrates into the cerebrospinal fluid (concentrations ranged from 35.3 to 119 ng/ml).

In conclusion, a validated assay for the quantitative determination of lamivudine in human plasma, saliva, and cerebrospinal fluid samples has been described. The assay meets the current requirements as to the validation of a bioanalytical methodology and can be used for pharmacokinetic studies with lamivudine in HIV infected patients.

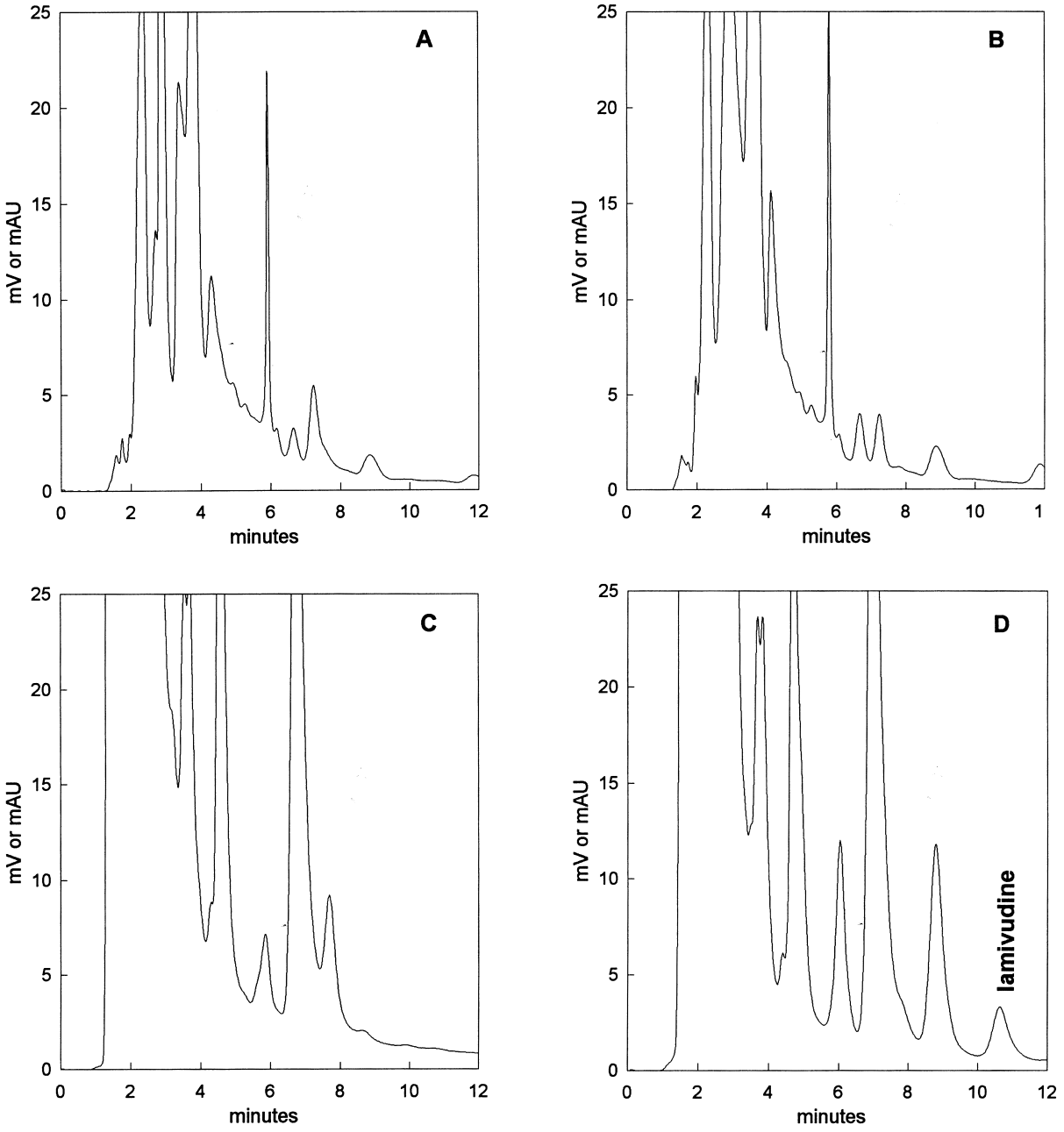

Fig. 2. Chromatograms of blank saliva (A), blank cerebrospinal fluid (B), blank plasma (C), and a plasma sample from a patient (527 ng/ml lamivudine) (D). The patient concomitantly used zidovudine (AZT).

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Table 1
Accuracy and precision for the analysis of lamivudine in human plasma

Concentration (ng/ml)	Accuracy (%)	Precision (%)		n[a]
		Between-day	Within-day	
100	97	9.0	3.5	8
1500	94	5.2	5.6	8
4000	95	6.2	3.7	8

[a] n=number of replicates.

Table 2
Stability of lamivudine in spiked human plasma samples

Storage conditions	Concentration (ng/ml)	Recovery (%)	R.S.D.[a] (%)	n[b]
1 h at 60°C	100	106	3.6	6
	1500	93	2.7	5
	4000	91	1.8	6
24 h at 25°C	100	99	2.8	6
	1500	95	2.7	6
	4000	97	0.9	6
7 days at 4°C	100	100	3.3	6
	1500	95	2.7	6
	4000	93	1.7	6
30 days at −30°C	100	98	1.9	6
	1500	96	3.1	6
	4000	102	2.0	6
3 freeze–thaw cycles	100	103	3.5	6
	1500	100	2.2	6
	4000	104	2.0	6

[a] R.S.D.=relative standard deviation.
[b] n=Number of replicates.

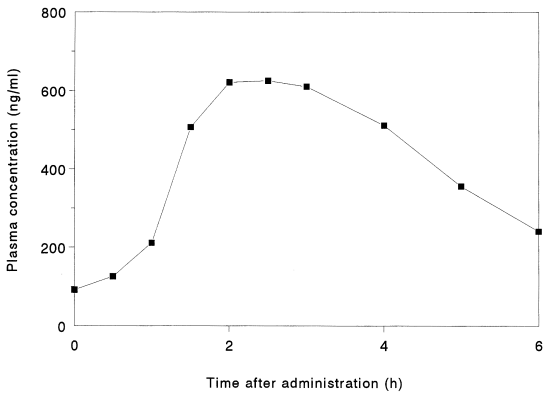

Fig. 3. Plasma concentration versus time curve of lamivudine after oral administration of 150 mg to an HIV-1 infected patient (chronic use). The patient concomitantly used zidovudine (AZT).

reference compound. Furthermore, R. van Gijn and H. Rosing are kindly acknowledged for their technical assistance and suggestions.

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