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Quantitative determination of abacavir (1592U89), a novel nucleoside reverse transcriptase inhibitor, in human plasma using isocratic reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

Abacavir is a novel nucleoside reverse transcriptase inhibitor for the treatment of HIV-1 infection. A simple and rapid high-performance liquid chromatographic method for the quantification of abacavir in human plasma suitable for pharmacokinetic research purposes is described. Sample pretreatment consists of protein precipitation with perchloric acid. The supernatant is injected directly into the chromatographic system after centrifugation. The drug is separated from endogenous compounds by isocratic reversed-phase high-performance liquid chromatography with ultraviolet detection at 285 nm. The method has been validated over the range of 20–2000 ng/ml using a volume of 300 μl of plasma. The assay is linear over this concentration range as indicated by the *F*-test for lack-of-fit. Within- and between-day precisions are less than 7.5% for all quality control samples. The lower limit of quantitation is 20 ng/ml and the recovery of abacavir is 88.1% (±1.3%). Frequently coadministered drugs did not interfere with the described methodology. Abacavir is stable in human plasma under various relevant storage conditions, for example when stored for 51 days at −20°C. This validated assay is suited for use in pharmacokinetic studies with abacavir in human plasma and can readily be implemented in the setting of a hospital laboratory for the monitoring of abacavir concentrations.

Keywords: Abacavir

1. Introduction

Abacavir (1592U89, Fig. 1) belongs to the class of the nucleoside reverse transcriptase inhibitors. The agent is anabolised by a unique intracellular mechanism to form carbovir triphosphate, which potently and selectively inhibits human immunodeficiency virus (HIV) reverse transcriptase [1]. Abacavir inhibits HIV-1-isolates from antiretroviral drug-naive patients with similar potency as the first available nucleoside analogue zidovudine [2].

Resistance to abacavir develops rather slowly. Cross-resistance between abacavir and didanosine or lamivudine, but not zidovudine or stavudine, has been reported in vitro. Abacavir has good oral

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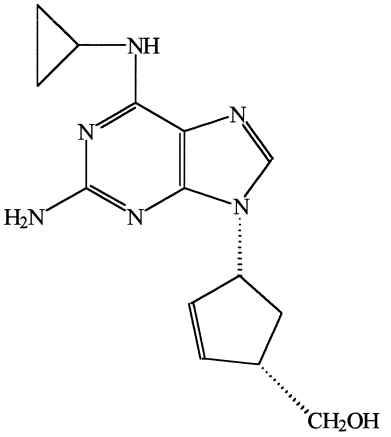

Fig. 1. Molecular structure of abacavir.

bioavailability and significantly penetrates the cerebrospinal fluid [3].

In vitro results suggest that abacavir does not significantly inhibit the human liver microsomal cytochrome (CYP) P450 isoenzymes 3A4, 2C9 or 2D6 activity and is unlikely to interact with compounds that are metabolised by these enzymes [4]. Treatment with abacavir in combination with other antiretroviral drugs (zidovudine, lamivudine, nevirapine, amprenavir and other protease inhibitors), decreased plasma HIV-1 RNA levels and increased CD4+ cell counts in patients with HIV-1 infection. In these studies, effectiveness was maintained for at least 48 weeks [5,6]. The currently recommended dosage for abacavir is 300 mg twice a day.

We here report the development and validation of a high-performance liquid chromatographic assay with ultraviolet detection for the quantitative determination of abacavir in human plasma. To our knowledge, no detailed methodology for the quantitative determination of abacavir in human plasma has been described thus far. The presented assay is useful for pharmacokinetic research in HIV-infected individuals treated with abacavir.

2. Experimental

2.1. Equipment

The high-performance liquid chromatography (HPLC) system consisted of a P580 isocratic pump (Gynkotek HPLC, Germering, Germany), a Basic+ Marathon autosampler, equipped with a 7739-005 injection valve (Rheodyne, Cotati, CA, USA), and a Spectroflow 757 absorbance detector (Kratos Analytical, Westwood, NJ, USA). The analytical column was a Symmetry C_{18} column (100×4.6 mm I.D., particle size 3.5 μm; Waters Chromatography, Milford, MA, USA) protected by a Symmetry C_{18} guard column (20×3.8 mm I.D.; Waters Chromatography). The column was thermostated in a waterbath and the temperature was controlled at 41±2°C using a Thermomix 1420 heating device (B. Braun, Melsungen, Germany). The analytical runs were processed by the Chromelion chromatographic data system (Gynkotek HPLC).

2.2. Chemicals

Abacavir was kindly provided by Glaxo Wellcome Research and Development (Stevenage, UK). Acetonitrile (gradient grade) and methanol (HPLC supragradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Phosphoric acid (85%, w/w) was purchased from Baker (Deventer, The Netherlands). Sodium hydroxide (analytical grade) was supplied by Merck (Darmstadt, Germany) and perchloric acid (>70%, w/w) by Acros Organics (Geel, Belgium). Water was laboratory-purified by reversed osmosis on a multi-laboratory scale. 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).

2.3. Preparation of standards, calibration samples and quality control samples

Stock solutions of abacavir were prepared by dissolving the appropiate amount of the compound, accurately weighed, in methanol to yield a concentration of approximately 160 or 100 μg/ml. For the preparation of calibration samples the stock solution containing approximately 100 μg/ml of abacavir was diluted with blank human plasma to 2000 ng/ml. Duplicate dilutions of 300 μl of the 2000 ng/ml solution of abacavir in plasma were made to yield abacavir concentrations in plasma of

20, 40, 100, 200, 400 and 1000 ng/ml. These calibration concentrations were freshly made before each analytical run.

The second and independently prepared stock solution (160 μg/ml), with separate weighing of the analyte, was used for the preparation of quality control samples (QCs) yielding concentrations of 50, 300 and 2000 ng/ml of abacavir in human plasma.

2.4. Sample pretreatment

A 300-μl aliquot of each sample was mixed with 75 μl of a 20% (w/w) solution of perchloric acid for 30 s. After centrifugtion for 15 min at 1300 *g*, 250 μl of the clear supernatant was transferred to an autosampler vial with insert.

2.5. Chromatography

The chromatographic analysis was performed at a temperature of 41±2°C on the previously described C_{18} analytical column with a mobile phase composed of phosphate buffer (25 m*M*, pH 7.0)–acetonitrile (85:15, v/v). Prior to use, air was removed by ultrasonication. Aliquots of 100 μl were injected using a flushed loop, the flow-rate was maintained at 1.0 ml/min and the absorbance was measured at 285 nm.

2.6. Specificity and selectivity

The interference of endogenous compounds was investigated by the analysis of different blank plasma samples originating from six individuals. The following compounds, frequently used by HIV-infected individuals, or their metabolites, were investigated for interference with the analytical method: adefovir, 3′-amino-3′-deoxythymidine, amprenavir, delavirdine, didanosine, efavirenz, fluconazole, folinic acid, ganciclovir, indinavir, itraconazole, lamivudine, methadone, nelfinavir, nevirapine, oxazepam, pyrazinamide, pyrimethamine, ranitidine, rifampin, ritonavir, saquinavir, stavudine, sulfamethoxazole, trimethoprim, zalcitabine, zidovudine and zidovudine-glucuronide in a final concentration of 20 μg/ml in mobile phase.

2.7. Limit of quantitation

The lower limit of quantitation (LLQ) was defined as the concentration for which the relative standard deviation and the deviation from the nominal concentration were both less than 20%. The upper limit of quantitation (ULQ) was arbitrarily set at 2000 ng/ml.

2.8. Accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precisions of the method were determined by assaying six replicates of each of the QCs in three separate analytical runs. The accuracy was calculated at each test concentration and was obtained by dividing the measured concentration by the nominal concentration and multiplying with 100%. The within-day and between-day precision were obtained by analysis of variance (ANOVA) for each test 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 weighing factor of $1/\text{concentration}^2$ [7,8]. For the construction of each calibration curve seven spiked plasma samples were analyzed in duplicate.

For the determination of the extraction yield, calibration samples of abacavir in previously deproteinized plasma (with perchloric acid) made from the same stock as the calibration samples in plasma, were used in the range of 20 to 2000 ng/ml. The recovery was calculated from these two calibration curves in two separate analytical runs by dividing the slopes of the calibration lines.

2.9. Stability

Blank plasma samples were spiked with appropriate aliquots of diluted abacavir stock solution to yield concentrations of 50, 300 and 2000 ng/ml. These samples were kept for 4.5 h at 60°C, 6.5 h at 37°C, three days at −20°C including four freeze–thaw cycles, three days at 25°C, 51 days at −20°C, and 51 days at −80°C. For each concentration and each storage condition three replicates were analyzed in one analytical run. The concentration of abacavir after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

The stability of the samples in the autosampler was tested by preparing a duplicate series of calibration samples. The second sample at each level was injected 24 h after the first, which was immediately injected on the column.

2.10. Analysis of patient samples

Plasma samples of 10 different HIV-1-individuals treated with abacavir (300 mg bid), and drawn at random times after the ingestion of the drug, were analyzed with the currently reported method.

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 or less.

3. Results and discussion

3.1. Chromatography and detection

Using the described C_{18} column, and a pH value of 7.0 of the mobile phase, abacavir showed sufficient retention and was also separated from the most prominent endogenous compounds. The UV spectrum of abacavir shows three maxima (217, 260 and 285 nm). A detection wavelength of 285 nm was chosen, as this is the most selective. Typical chromatograms of a blank plasma sample, a spiked sample and a patient sample are shown in Fig. 2. The runtime of the assay is 15 min. Due to a rapid decline of the number of theoretical plates after 50 injections, the pre-column had to be replaced after approximately 60 injections of the perchloric acid extracts.

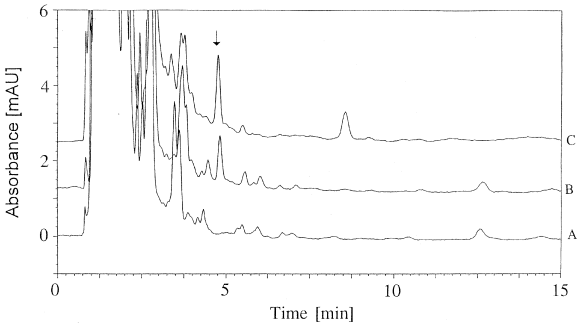

Fig. 2. Chromatograms typical for the analysis of abacavir (blank plasma) (A), a spiked plasma sample of 67 ng/ml (B), and a patient sample containing 92 ng/ml abacavir (C) (t_r=4.8 min).

3.2. Sample pretreatment and recovery

In order to obtain a bio-analytical assay for abacavir with a lower limit of quantification in the range of 10 to 50 ng/ml, a simple procedure was preferred. The method development was therefore started, after an appropriate chromatographic analytical system was found, by testing protein precipitation with direct injection. When trichloroacetic acid, perchloric acid and acetonitrile as precipitation agents were compared, perchloric acid was chosen because of a higher recovery compared to trichloroacetic acid and the lower dilution factor compared to acetonitrile. Approximately 99% of the plasma proteins are precipitated using the current method with perchloric acid [9].

Protein precipitation with perchloric acid followed by direct injection into the HPLC system is a rapid, simple and effective sample pretreatment procedure. Recovery of abacavir from spiked human plasma samples using this method is 88.1% (±1.3%) over the whole tested concentration range.

3.3. Specificity and selectivity

Blank, drug-free plasma from six different individuals showed no interfering endogenous substances eluting at the retention time (t_r) of abacavir. Potentially co-administered drugs or metabolites tested had retention times that were different from abacavir (t_r=4.8 min) (3′-amino-3′-deoxythymidine t_r=1.3 min, didanosine t_r=1.6 min, folic acid t_r=1.1 min, ganciclovir t_r=1.2 min, lamivudine t_r=1.6 min, nevirapine t_r=14.2 min, pyrazinamide t_r=1.6 min, ranitidine t_r=3.8 min, stavudine t_r=1.8 min, sulfamethoxazole t_r=2.2 and 8.8 min, trimethoprim t_r= 8.8 min, zidovudine t_r=3.8 min, and zidovudine glucuronide t_r=1.4 and 1.5 min) or were not detected with the described analytical method.

Table 1
Accuracy and precision for the analysis of abacavir in spiked human plasma samples

Concentration (ng/ml)	Accuracy (%)	Between-day precision	Within-day precision	n[a]
50	100.2	7.5	7.5	18
300	102.1	1.7	2.7	18
2000	97.6	0.3	0.7	18

[a] n: Total number of replicates in three analytical runs.

3.4. Limit of quantitation

The relative standard deviation and deviation from the nominal concentration were both less than 20% at a concentration of 20 ng/ml in plasma. At all other concentrations up to the ULQ (2000 ng/ml), the relative standard deviation and percent deviation from the nominal concentration were always less than 15%. Therefore, we defined 20 ng/ml as the LLQ.

In clinical practice, the average steady-state peak concentration is up to 2 μg/ml [2]. Hence, the concentration range which is required for the application of this HPLC method in clinical pharmacokinetic studies is covered.

3.5. Accuracy, precision, linearity and stability

No suitable internal standard was available. However, the assay provided satisfactory validation results without using an internal standard. Calibration curves proved to be linear in the range of 20 to 2000 ng/ml with the use of the F-test for lack-of-fit ($\alpha = 0.05$) as an indicator of linearity of the regression model. Accuracies and precisions for the quantitation of abacavir in human plasma are listed in Table 1. The use of peak areas in combination with a weighing factor ($1/\text{concentration}^2$) resulted in a minimal deviation from nominal concentrations. The method proved to be accurate (average accuracy at three concentrations 97.6–102.1% of the nominal concentrations) and precise (within-day precision ranged from 0.7 to 7.5% and between-day precision

Table 2
Stability of abacavir in spiked human plasma samples

Storage condition	Concentration (ng/ml)	Recovery (%)	Standard deviation (%)	n
4.5 h at 60°C	50	93[a]	21	3
	300	94	1	3
	2000	95	2	3
6.5 h at 37°C	50	99[a]	28	3
	300	95	2	3
	2000	96	1	3
3 days at 25°C	50	122[a]	35	3
	300	96	2	3
	2000	97	1	3
3 days at −20°C including 4 freeze–thaw cycles	50	105	5	3
	300	96	0	3
	2000	96	1	3
51 days at −20°C	50	115	5	4
	300	99	2	4
	2000	100	0	4
51 days at −80°C	50	110	10	3
	300	101	1	3
	2000	100	1	3

[a] A small interfering peak was observed and not always completely separated from abacavir.

ranged from 0.3 to 7.5%). Correlation coefficients (r^2) of calibration curves were >0.998 as determined by least-squares linear regression analysis.

Under all conditions tested abacavir is stable with concentrations of at least 93% of the initial concentrations. Unfortunately, there is a rather large standard deviation for the lowest concentration (50 ng/ml). This was probably caused by a small interfering peak, which was not always separated from abacavir (Table 2). Abacavir is stable when kept in the autosampler for 24 h (recovery 97.3%).

3.6. Analysis of patient samples

The applicability of the assay for pharmacokinetic research in HIV-1-infected individuals was demonstrated by measuring 10 patient samples obtained at different time points after ingestion of 300 mg abacavir. In this population we found plasma concentrations between 29 and 1556 ng/ml.

4. Conclusion

In conclusion, a sensitive, specific and validated assay for the quantitative determination of abacavir in human plasma is described. The applicability of the assay for pharmacokinetic research in HIV-1-infected individuals is demonstrated with the analysis of plasma samples from HIV-1-infected patients. The assay meets all current requirements for the validation of a bioanalytical method [7]. This HPLC assay can be used for pharmacokinetic studies with abacavir in HIV-1-infected individuals.

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