BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **18**: 862–865 (2004)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.406

ORIGINAL RESEARCH

Determination of abacavir in human plasma by high-performance liquid chromatography with ultraviolet detection and the analytical error function

Salut M. Ferrer, Pilar Modamio, Cecilia F. Lastra and Eduardo L. Mariño*

Clinical Pharmacy and Pharmacotherapy Unit, Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain

Received 26 February 2004; revised 26 April 2004; accepted 29 April 2004

ABSTRACT: A rapid and simple high-performance liquid chromatography method has been developed for the determination of the HIV-1 reverse transcriptase inhibitor abacavir in human plasma. It included a single liquid–liquid extraction procedure with a mixture of ethyl acetate-diethyl ether prior to reversed-phase chromatography on a C_{18} column and C_{18} precolumn insert. Ultraviolet detection was set at 285 nm. The mobile phase consisted of water–acetonitrile (83:17, v/v) and the flow rate was kept at 1 mL/min. The total run time for a single analysis was 10 min. The method has been validated over the range 50–2500 ng/mL. The assay was linear over the entire concentration range ($r^2 = 0.9993$). Intra- and inter-day precision and accuracy were less than 8.1 and −5.2%, respectively. The extraction recovery was greater than 94.3%. Abacavir was stable under the relevant storage conditions tested. After the validation, the analytical error function was established as standard deviation (SD; ng/mL) = −1.072 + 0.037*C* (*C* = theoretical concentration value). The method developed and its associated analytical error function will be suitable for pharmacokinetic studies and monitoring of HIV-1 patients. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: Abacavir; HPLC; human plasma; analytical error function

INTRODUCTION

Abacavir is a 2′-deoxyguanosine analog (Fig. 1) and a potent *in vitro* and *in vivo* HIV-1 reverse transcriptase inhibitor (Daluge *et al.*, 1997). Many favourable characteristics led to its accelerated incorporation into antiretroviral multidrug therapies such as high bioavailability after oral administration not affected by food (Chittick *et al.*, 1999), which allows easier regimens that help patients maintain adherence (Clumeck *et al.*, 2001; Opravil *et al.*, 2002). Although abacavir is well tolerated in general, severe adverse effects such as hypersensitivity reaction may cause discontinuation and require a careful follow-up of the patient (Clay, 2002; Hetherington *et al.*, 2001; Peyriere *et al.*, 2003).

To our knowledge, different methods for the simultaneous determination of antiretroviral drugs in human plasma by HPLC with ultraviolet detection include abacavir (Aymard *et al.*, 2000; Simon *et al.*, 2001), and there are also few specific methods for abacavir alone (Veldkamp *et al.*, 1999; Weller *et al.*, 2000). In all of them sample pretreatment consisted of solid phase extraction procedure or protein precipitation. Other HPLC methods determined abacavir and its metabolites in other biological matrices such as cell extracts, urine and cerebrospinal fluid (Fung *et al.*, 2001; Ravitch and Moseley, 2001). None of the published studies reported the error function associated with the analytical method.

Thus, the goal of this study was first to develop and validate a rapid HPLC method using a simple liquid–liquid extraction procedure for the quantitation of abacavir in human plasma, which would be appropriate for future monitoring of HIV-1 patients. Secondly, to establish the analytical error function of the method

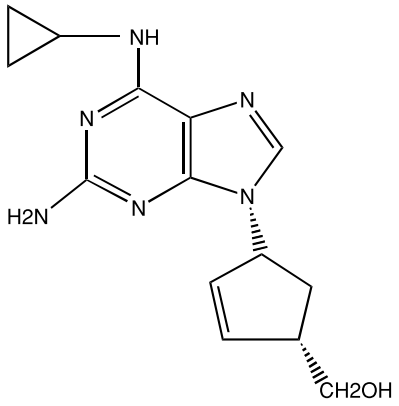

Figure 1. Molecular structure of abacavir.

*Correspondence to: E. L. Mariño, Clinical Pharmacy and Pharmacotherapy Unit, Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain.
E-mail: emarino@ub.edu

Abbreviations used: HPLC, high-performance liquid chromatography.

Contract/grant sponsor: Fundación Española de Farmacia Hospitalaria.
Published online 6 September 2004

in order to provide a possible weighing method for nonlinear regression estimation of the pharmacokinetic parameters.

EXPERIMENTAL

Chemicals and reagents. Abacavir was kindly supplied by GlaxoSmithKline (Stevenage, UK). Diethyl ether and ethyl acetate (analytical grade) were purchased from Panreac (Montcada i Reixach, Barcelona, Spain) and Carlo Erba (Barcelona, Spain), respectively. HPLC-grade acetonitrile was purchased from Carlo Erba (Barcelona, Spain). Water was deionized and osmosed by a Milli-Q Plus system (Millipore, Barcelona, Spain). Blank drug-free human plasma was obtained from the Hospital General of Granollers blood bank (Granollers, Barcelona, Spain).

Instrumentation. The HPLC system consisted of a Kontron Model 420 Liquid Chromatograph (Kontron Instruments, Barcelona, Spain) equipped with an automatic sampling system with a variable volume injector (model 465), two pumps (model 420), a mixer (model 491), a capillary UV–visible detector with variable wavelength (model 433) and a computerized integration system data output (model MT-450).

Chromatographic conditions. Reversed-phase liquid chromatography was carried out at room temperature using a Luna C_{18} analytical column, 5 µm, 150 × 4.6 mm i.d. (Phenomenex, Barcelona, Spain) protected by a C_{18} (ODS Octadecyl), 4 × 3.0 mm i.d. in-line guard column (Phenomenex, Barcelona, Spain). The ultraviolet detector was set to monitor the 285 nm wavelength. Isocratic separation was performed at a flow-rate of 1.0 mL/min. The mobile phase consisted of HPLC-grade water and acetonitrile (83:17 v/v). Prior to use, air was removed by ultrasonication.

Preparation of stock solutions and standards. Stock solutions of abacavir were prepared by dissolving accurately weighed amounts of drug powder in HPLC water to yield 200 µg/mL drug concentration. Stock solutions were stored at −25 ± 1°C until use. Calibration standards covering the range from 50 to 2500 ng/mL were made in drug-free human plasma by further dilution of stock solution in blank human plasma. For the preparation of quality control standards (250, 1000 and 2500 ng/mL) used in the validation, the same procedure was followed.

Sample extraction procedure. A single liquid–liquid extraction procedure was performed. One milliliter of human plasma was transferred to a 10 mL glass tube. Eight milliliters of ethyl acetate–diethyl ether (75:25 v/v) were added. The mixture was shaken for 10 min on a rotary mixer. The organic phase was then transferred to a conical glass tube and evaporated to dryness under a gentle stream of nitrogen at 40 ± 1°C. The residue was reconstituted on 140 µL of mobile phase and 80 µL were injected into the column.

Validation procedure. Evaluation of the HPLC method was based on linearity, precision, accuracy, recovery and stability assays (Causon, 1997; Karnes *et al.*, 1991; Shah *et al.*, 1992, 2000).

Linearity was determined using the same concentration range as the calibration curve (50, 100, 250, 1000 and 2500 ng/mL). Four replicate plasma samples of each concentration were analyzed.

Intra-day and inter-day precision and accuracy were estimated by assaying six replicate plasma samples at three different concentrations (quality control standards) within the linearity range on the same day (intra-day assay) and five separate days (inter-day assay).

The absolute recovery (extraction efficiency) of abacavir in human plasma was determined by comparing the peak areas obtained from the plasma extracts with the standard stock solutions, across the range of calibration. Four replicates of each different concentration were assayed.

The limit of quantitation (LOQ) was defined as the lowest concentration for which precision and accuracy were less than 20% and was measured at a signal-to-noise ratio of 7:1.

To determine the influence of temperature on the stability of abacavir, quality control standards were stored at different temperatures. Since HIV-infected patient samples are routinely heated at 57 ± 1°C to inactivate the virus prior to handling, heat deactivation studies were performed to ensure the stability of the compound. Quality control standards were also subjected to three freeze–thaw cycles. For each concentration and storage condition three replicates were analysed in one analytical run. The concentration of abacavir after each storage period was related to the concentration of samples freshly prepared and immediately processed.

Analytical error function. The procedure used to obtain the error function of the method previously validated was the analysis of five calibration curves on separate days (four replicates each concentration). The mean and standard deviation (SD) of each standard concentration were obtained from each daily calibration curve. The best function between SD and the theoretical standard concentration values (C) were calculated by means of multiple regression. The stepwise forward selection method was applied (Modamio *et al.*, 1996).

RESULTS AND DISCUSSION

Sample preparation and recovery

In order to obtain a simple and effective analytical method, many pretreatment procedures were assayed. Protein precipitation was tested in our laboratory, showing poor results despite of effectiveness in previously described methods for abacavir (Veldkamp *et al.*, 1999; Weller *et al.*, 2000). On the other hand, different methods showed that other nucleoside reverse transcriptase inhibitors could be isolated from plasma by liquid–liquid extraction (Hedaya and Sawchuk, 1988; Moyer *et al.*, 1999; Unadkat *et al.*, 1988; Wientjes and Au, 1991). Therefore various liquid–liquid extraction procedures were assayed. The presented extraction was chosen because it reliably eliminated interfering

material from plasma, with higher recovery of abacavir. The absolute extraction recoveries for the range of concentrations studied were greater than 94.3%.

Chromatogram

Representative chromatograms of a blank plasma (a) and plasma spiked with 500 ng/mL abacavir (b) are presented in Fig. 2(a,b). There were no interfering peaks in drug-free plasma eluting at the retention time of interest, which was approximately 6.1 min. Besides, no interference was expected according to a specific reversed-phase HPLC method with UV detection developed to quantify abacavir plasma concentrations (Veldkamp *et al.*, 1999). The run time of the assay was 10 min.

Assay validation

Calibration curves proved to be linear in the range of 50–2500 ng/mL. In the linearity assay the response factor expressed by the percentage coefficient of variation (%CV) was 4.3%. The regression equation obtained by unweighted least-squares linear regression was $y = 0.016 + 0.009x$, where y is peak area and x is concentration. The correlation coefficient was $r^2 = 0.9993$. The standard error of the slope was 5.1×10^{-5} and F-ratio was 30,324.8. Therefore, it can be stated that a good linear relationship between the peak area and concentration over the calibration range was found.

Precision was expressed as the percentage of coefficient of variation at each concentration (%CV). Accuracy was calculated as the percentage deviation from the nominal concentration (%Bias). Precision and accuracy results for the quantitation of abacavir in human plasma are listed in Table 1. Maximum CV value was 8.1% in intra-day precision for the highest concentration (2500 ng/mL). The highest bias (–5.2%) was found in the inter-day accuracy for the lowest concentration (250 ng/mL).

The LOQ was established in 50 ng/mL. Abacavir was stable for at least 24 h at room temperature (95.5% recovery). Samples kept for 1 month at –25 ± 1°C were stable with concentrations of 96.1% of the initial concentrations. After three freeze–thaw cycles, recovery was 100.4%. Finally, samples kept for 1 h at 57 ± 1°C showed a recovery of 95.9%.

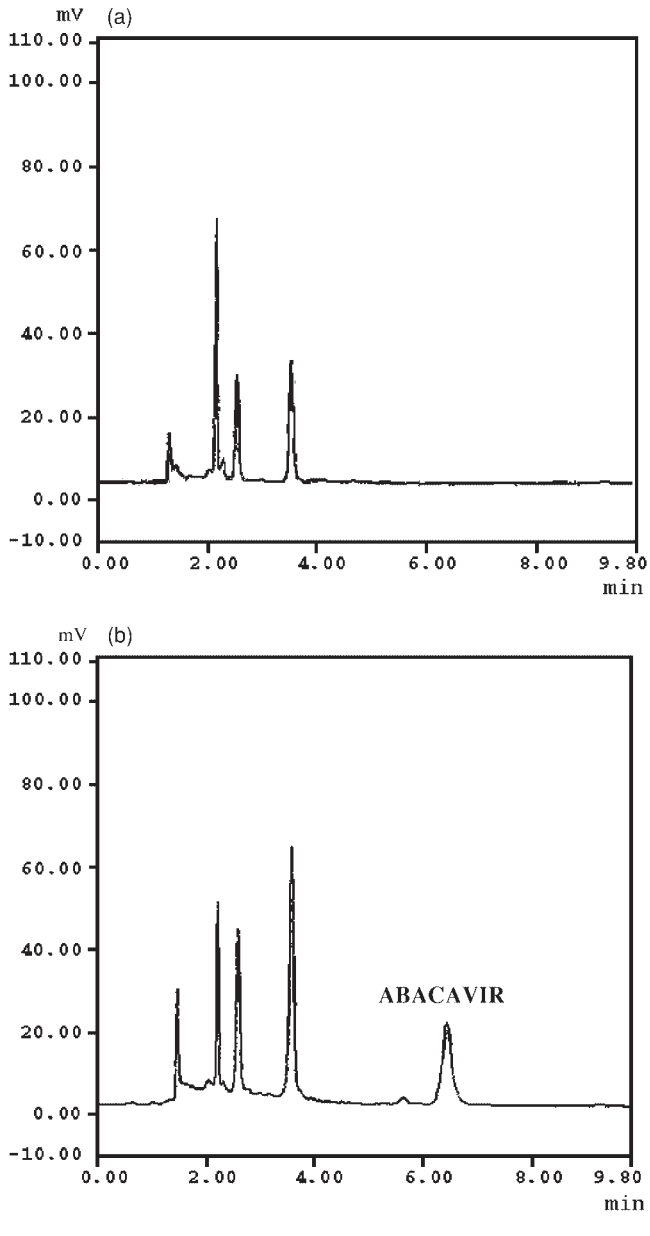

Figure 2. Chromatograms of blank plasma (a) and plasma spiked with 500 ng/mL of abacavir (b).

Analytical error function

The selection of the analytical error function was made by the stepwise forward procedure. This discriminates

Table 1. Intra- and inter-day precision and accuracy for abacavir in human plasma

Spiked concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 30)		
	Concentration found (ng/mL)	CV(%)	Bias(%)	Concentration found (ng/mL)	CV(%)	Bias(%)
250	248.4	6.5	0.7	262.9	6.2	−5.2
1000	1029.8	7.2	−3.0	997.6	5.1	−0.2
2500	2471.1	8.1	1.2	2487.8	5.9	0.5

CV = (SD/mean) × 100; bias(%) = [(concentration found − spiked concentration)/spiked concentration] × 100.

the best fitting according to coefficient of correlation, standard deviation, F, and probability. The analytical error function which fits best the results was linear and its equation was: SD (ng/mL) = $-1.072 + 0.037C$. The knowledge of the analytical error function could be used as an alternative to the classic weighting methods (1, $1/C$, $1/C^2$) in the pharmacokinetic treatment of the plasma levels of abacavir by nonlinear regression analysis (Jansat *et al.*, 1998).

CONCLUSIONS

The method developed is a simple, rapid and reliable procedure for the analysis of abacavir in human plasma, meeting all the requirements for the validation of an analytical methodology. It is adequate to monitor patients receiving therapeutic dosages of the drug since plasma concentrations achieved for the currently recommended dosage for abacavir (300 mg twice a day) according to the literature reviewed (C_{max} of 2170 ng/mL) are within the working range of our method (Weller *et al.*, 2000). The analytical error function established provides a suitable data weighting method covering the working range selected.

Acknowledgments

The authors are grateful to Fundación Española de Farmacia Hospitalaria for financial support. This work has been also possible with the support of the Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya for granting a research fellowship. The authors would like to express their gratitude to GlaxoSmithKline for kindly providing the drug, and the Hospital General de Granollers for supplying the drug-free plasma.

REFERENCES

Aymard GM, Legrand M, Trichereau N and Diquet B. Determination of twelve antiretroviral agents in human plasma sample using reversed-phase high performance liquid chromatography. *Journal of Chromatography B* 2000; **744**: 227–240.

Causon R. Validation of chromatographic methods in biomedical analysis. Viewpoint and discussion. *Journal of Chromatography B* 1997; **689**: 175–180.

Chittick GE, Gillotin C, McDowell JA, Lou Y, Edwards KD, Prince WT and Stein DS. Abacavir (1592U89): absolute bioavailability and effect on food. *Pharmacotherapy* 1999; **19**: 932–942.

Clay PG. The abacavir hypersensitivity reaction: a review. *Clinical Therapy* 2002; **10**: 1502–1514.

Clumeck N, Goebel F, Rozenbaum W, Gerstoft J, Staszewski S, Montaner J, Johnson M, Gazzard B, Stone C, Athisegaran R, Moore S and CNA30017 Study Team. Simplification with abacavir-based triple nucleoside therapy versus continued protease inhibitor-based highly active antiretroviral therapy in HIV-1-infected patients with undetectable plasma HIV-1 RNA. *AIDS* 2001; **15**: 1517–1526.

Daluge SM, Good SS, Faletto MB, Miller WH, St Clair MH, Boone LR, Tisdale M, Parry NR, Reardon JE, Dornsife RE, Averett DR and Krenitsky TA. 1592U89, a novel carbocyclic nucleoside analog with potent, selective anti-human immunodeficiency virus activity. *Antimicrobial Agents and Chemotherapy* 1997; **41**: 1082–1093.

Fung EN, Cai Z, Burnette TC and Sinhababa AK. Simultaneous determination of Ziagen and its phosphorilated metabolites by ion-pairing high-performance liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B* 2001; **754**: 285–295.

Hedaya MA and Sawchuk RJ. A sensitive liquid-chromatographic method for determination of 3′-azido-3′-deoxythymidine (AZT) in plasma and urine. *Clinical Chemistry* 1988; **34**: 1565–1568.

Hetherington S, McGuirk S, Powell G, Cutrell A, Naderer O, Spreen B, Lafon S, Pearce G and Steel H. Hypersensitivity reactions during therapy with the nucleoside reverse transcriptase inhibitor abacavir. *Clinical Therapy* 2001; **23**: 1603–1614.

Jansat JM, Lastra CF and Mariño EL. Comparative study of different weighting methods in non-linear regression analysis: implications in the parametrization of carebastatine after intravenous administration in healthy voluntees. *International Journal of Clinical and Phamacological Therapy* 1998; **36**: 340–344.

Karnes HT, Shiu G and Shah V. Validation of bioanalytical methods. *Pharmaceutical Research* 1991; **8**: 421–426.

Modamio P, Lastra CF and Mariño EL. Determination of analytical error function for β-blockers as a possible weighting method for the estimation of the regression parameters. *Journal of Pharmaceutical and Biomedical Analysis* 1996; **14**: 401–408.

Moyer TP, Temesgen Z, Enger R, Estes L, Charlson J, Oliver L and Wright A. Drug monitoring of antiretroviral therapy for HIV-1 infection: method validation and results of a pilot study. *Clinical Chemistry* 1999; **45**: 1465–1476.

Opravil M, Hirschel B, Lazzarin A, Furrer H, Chave JP, Yerly S, Bisset LR, Fischer M, Vernazza P, Bernasconi E, Battegay M, Ledergerber B, Gunthard H, Howe C, Weber R, Perrin L and Swiss HIV Cohort Study. A randomised trial of simplified maintenance therapy with abacavir, lamivudine, and zidovudine in human immunodeficiency virus infection. *Journal of Infectious Diseases* 2002; **185**: 1251–1260.

Peyriere H, Guillemin V, Lotthe A, Baillat V, Fabre J, Favier C, Atoui N, Hansel S, Hillaire-Buys D and Reynes J. Reasons for early abacavir discontinuation in HIV-infected patients. *Annals of Pharmacotherapy* 2003; **37**: 1392–1397.

Ravitch JR and Moseley CG. High performance liquid chromatographic assay for abacavir and its two major metabolites in human urine and cerebrospinal fluid. *Journal of Chromatography B* 2001; **762**: 165–173.

Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Viswanathan CT, Cook CE, McDowall RD, Pittman KA and Spector S. Analytical methods validation: bsioavailability, bioequivalence and pharmacokinetic studies. *Pharmaceutical Research* 1992; **9**: 588–592.

Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ, McKay G, Miller KJ, Patnaik RN, Powell ML, Tonelli A, Viswanathan CT and Yacobi A. Bioanalytical method validation: a revisit with a decade of progress. *Pharmaceutical Research* 2000; **17**: 1551–1557.

Simon VA, Thiam MD and Lipford LC. Determination of serum levels of thirteen human immunodeficiency virus-suppressing drugs by high-performance liquid chromatography. *Journal of Chromatography A* 2001; **913**: 447–453.

Unadkat JD, Crosby SS, Wang JP and Hertel CC. Simple and rapid high-performance liquid chromatographic assay for zidovudine (azidothymidine) in plasma and urine. *Journal of Chromatography* 1988; **430**: 420–423.

Veldkamp AI, Sparidans RW, Hoetelmans RMW and Beijnen JH. 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. *Journal of Chromatography B* 1999; **736**: 123–128.

Weller S, Radomsky KM, Lou Y and Stein DS. Population pharmacokinetics and pharmacodynamic modelling of abacavir (1592U89) from a dose-ranging, double-blind, randomised monotherapy trial with human immunodeficiency virus-infected subjects. *Antimicrobial Agents and Chemotherapy* 2000; **44**: 2052–2060.

Wientjes MG and Au J. High-performance liquid chromatographic analysis of 2′,3′-dideoxyinosine in biological samples. *Journal of Chromatography* 1991; **563**: 407–413.

