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Journal of Chromatography B, 762 (2001) 165–173

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

High-performance liquid chromatographic assay for abacavir and its two major metabolites in human urine and cerebrospinal fluid

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Received 8 January 2001; received in revised form 13 June 2001; accepted 31 July 2001

Abstract

A simple, reversed-phase HPLC assay has been developed and validated to measure the HIV-1 reverse transcriptase inhibitor abacavir and its two major metabolites, a 5′-glucuronide and a 5′-carboxylate, in human urine and cerebrospinal fluid. Sample preparation involved centrifuging to minimize particulates, then diluting the supernatant before HPLC separation and ultraviolet detection at 295 nm. The method described was used successfully to measure concentrations of abacavir and its two major metabolites in urine and cerebrospinal fluid from HIV-1 infected subjects.

Keywords: Abacavir; Abacavir 5′-glucuronide; Abacavir 5′-carboxylate

1. Introduction

Abacavir (1592U, Ziagen, Fig. 1) is a nucleoside analog that is an HIV-1 reverse transcriptase inhibitor and a potent in vivo and in vitro inhibitor of HIV-1, the causative agent of the acquired immunodeficiency syndrome (AIDS) [1,2]. Accelerated approval applications to use abacavir in combination with other anti HIV-1 agents have been approved by the US Food and Drug Administration (FDA) and in the European Union, Canada, and Australia, among other countries, based on observed beneficial effects of abacavir on surrogate markers of HIV-1 activity.

The two major metabolites of abacavir in humans are a 5′-glucuronide conjugate and a 5′-carboxylate (361W and 2269W, respectively, Fig. 1). Many of

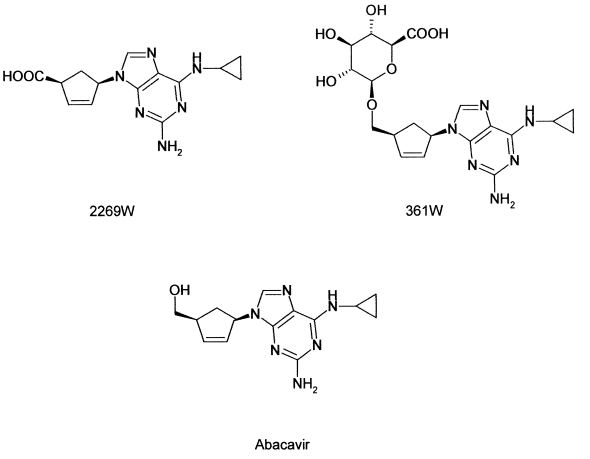

Fig. 1. Structures of abacavir and its two major metabolites, 2269W and 361W.

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00361-9

the potential co-therapies for HIV-1 such as HIV protease inhibitors are metabolized extensively by cytochrome P450 isozymes such as CYP3A4 [3,4], and are often inducers or inhibitors of the P450 isozymes, leading to a potential for drug–drug interactions [3,4]. In contrast, the formation of abacavir 5′-glucuronide and 5′-carboxylate are mediated by the enzymes uridine 5′-diphosphate glucuronyl transferase (UDPGT) and alcohol/aldehyde dehydrogenase (ADH/ALDH), respectively [5]. Because the enzymes involved in metabolizing abacavir are not thought to be major pathways of metabolism of potential co-administered therapies, drug–drug interactions with these therapies are theoretically unlikely. However, because of increasing concern about drug–drug interactions and their frequent association with loss of efficacy or increase in toxicity [6], studies of the potential for new entities such as abacavir to be involved in drug–drug interactions are of great interest, and the need for simple, high-throughput assays to measure concentrations of abacavir and its major metabolites in these studies is critical. Such an assay would also prove useful in characterizing the metabolism of abacavir in special patient groups, such as hepatic-impaired or ADH/ALDH deficient populations. Assays of cerebrospinal fluid concentrations of abacavir would be beneficial in determining the possible utility of this drug in treating central nervous system reservoirs of HIV-1 in AIDS patients.

This report describes the development and validation of assays of abacavir and its two major metabolites in human urine and cerebrospinal fluid, using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. These assays have been used to obtain pharmacokinetic data in HIV-1 infected subjects.

2. Experimental

2.1. Equipment

The HPLC system components for the assays were a Model 616S pump (Waters, Milford, MA, USA), a Model 717P autosampler (Waters), a Model 996 photodiode array detector (Waters), and a Model 600S controller (Waters). The HPLC column used was a Kromasil octadecyl column (150×3.2 mm; 5 μm particle size; Phenomenex, Torrance, CA, USA); the use of a guard column or on-line filter was not necessary. Chromatographic data were acquired and analyzed using MultiChrom software (version 2.21, Thermo LabSystems, Altrincham, UK).

2.2. Chemicals

Abacavir (Lot UAO), 361W (Lot UA), 2269W (Lot UB) were all supplied by Glaxo Wellcome. Methanol and acetonitrile (both HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). Glacial acetic acid (reagent grade) was purchased from Sigma–Aldrich (Milwaukee, WI, USA). Ammonium acetate (reagent grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium hydroxide 8% (w/v) solution (reagent grade), was obtained from VWR (Atlanta, GA, USA). Deionized water was produced using a Milli-Q Plus water purifier (Millipore, Molsheim, France). Control human cerebrospinal fluid was purchased from Sigma–Aldrich (St. Louis, MO, USA). Blank, drug-free urine was obtained from healthy male volunteers.

2.3. Preparation of analytical standard solutions

Analytical stock solutions of abacavir, 2269W, and 361W were prepared by dissolving the appropriate amount of each powder in water to yield a final concentration of approximately 0.5–1.0 mg/ml. In order to completely dissolve 361W, 15 μl of 8% (w/v) sodium hydroxide was added for each 5 ml of solution volume, and the stock was vortex-mixed. The 2269W dissolved easier in water if 10% (v/v) methanol was added prior to sonicating the solution gently. Analytical standard solutions for each analyte were only used when freshly prepared, to make the calibration and quality control stocks. Separate sets of analytical standard solutions were used to prepare calibration curve stocks and assay quality control stock solutions for each matrix.

In order to prepare urine or cerebrospinal fluid calibration standards and assay quality control samples, portions of each set of three analytical stock solutions were diluted in blank human urine or cerebrospinal fluid to give mixtures of the three

Table 1
Accuracy and precision of the assays for abacavir, 2269W and 361W in human urine

Spiked concentration (μg/ml)			Intra-assay precision (RSD, %)			Inter-assay precision (RSD, %)			Bias (%)		
361W	2269W	Abacavir	361W	2269W	Abacavir	361W	2269W	Abacavir	361W	2269W	Abacavir
0.723	0.336	0.787	1.87	1.52	1.60	0.86	0.61	1.06	−3.81	5.09	−3.81
8.68	4.40	9.44	2.27	2.18	2.28	1.40	0.88	1.05	0.85	2.60	1.33
44.9	22.7	48.8	0.77	0.75	0.55	2.01	2.29	1.56	2.31	4.07	3.00

Note: Bias is reported as mean percent difference from nominal concentration. n=15–20/group.

analytes at final concentrations of approximately 20 or 200 μg/ml. These calibration standard stock solutions and assay quality control stock solutions were stored at approximately −20°C between uses.

2.4. Sample preparation

Frozen study samples, calibration standard stock solutions and assay quality control stock solutions were thawed to room temperature on a laboratory benchtop, then vortex-mixed. Appropriate portions of the calibration standard stock solutions and assay quality control stock solutions were diluted in HPLC injection vials with mobile phase to a final volume of 750 μl and vortex-mixed, to create individual calibration standards and assay quality control samples over the analytical range of approximately 0.290–56.6 μg/ml. Each set of study samples assayed included at least duplicate sets of five separate calibration standard dilutions and three separate assay quality control sample dilutions (Tables 1 and 2). Portions of the study samples were centrifuged at approximately 12000 *g* for 5 min to minimize particulates, then 75 μl portions were transferred to HPLC injection vials and diluted with mobile phase to a final volume of 750 μl (1:10 dilution).

Concentrations of one or both metabolites of abacavir exceeded the upper limits of their respective calibration curves on occasion. In this situation, portions of the 1:10 diluted samples were diluted further with mobile phase to final concentrations of 1:50 or 1:100, and then re-assayed along with sets of calibration standards and assay quality control samples.

The use of an internal standard was not necessary with this assay, because sample processing involved simply diluting and chromatographing samples.

2.5. HPLC analysis

The standards and samples were chromatographed on a Kromasil octadecyl column at room temperature with mobile phase containing 25 m*M* ammonium acetate buffer (pH 4.0 with acetic acid)–methanol (95:5, v/v) initially, and a linear gradient of acetonitrile increasing from 0 to 50% over 30 min. A 10 min HPLC column re-equilibration time followed each individual analysis. The mobile phase flow-rate was 0.7 ml/min, and the total run time for each injection was 40 min. The analytes were quantitated following UV detection at 295 nm.

Table 2
Accuracy and precision of the assays for abacavir, 2269W, and 361W in human cerebrospinal fluid

Spiked concentration (μg/ml)			Precision (RSD, %)			Bias (%)		
361W	2269W	Abacavir	361W	2269W	Abacavir	361W	2269W	Abacavir
0.142	0.138	0.141	6.59	6.35	6.65	−7.75	−4.35	2.84
0.689	0.689	0.694	2.10[B]	1.31[B]	–[A]	7.40	9.29	13.8
4.32	4.32	4.35	0.99	1.33	1.15	−2.55	−1.85	4.37

Note: Bias is reported as mean percent difference from nominal concentration. n=3 or 4, except as noted. A=Not evaluated (n=1), B=(n=2).

2.6. Treatment of data

Data acquisition and integration were controlled using MultiChrom software (version 2.21, Thermo LabSystems). Concentrations of abacavir, 2269W, and 361W in each sample and calibration standard were determined using the parameter estimates from a linear least-squares regression analysis of the chromatographic peak area (''y'') on the spiked concentration of the calibration curve data for each analyte (''x''), with the weighting factor being $1/x^2$. Acceptance of each sequence of assays was based on evaluation of the calibration curve and assay quality control sample values in accordance with specificity, accuracy, and precision criteria described below. All statistical analyses and precision/bias estimates were calculated using Microsoft Excel software, version 5.0.

2.7. Assay specificity

Chromatographic interference from endogenous compounds was initially investigated using urine samples obtained from six healthy volunteers. Since abacavir is being administered to HIV^+ patients who are also being treated with many other drugs, the major focus of specificity studies involved the potential for assay interference from endogenous compounds and from co-therapies in subjects enrolled in studies of abacavir. Pre-dose urine and cerebrospinal fluid samples from these subjects were examined on a routine basis. The assay was considered to be specific if the mean size of any interfering peaks was not greater than 20% of the lower limit of quantitation (LLOQ) for the assay.

2.8. Accuracy, precision, and limits of quantitation

The accuracy of back-calculated standards and assay quality control samples was assessed as a percent difference from their stated nominal concentrations. At least 70% of the standards assayed for each set of samples must back-calculate to within $\pm 20\%$ of nominal. The interpolated assay quality control estimates had to be within 15% of nominal for all concentrations. At least 67% of the assay quality control samples within an analytical run were required to fall within acceptance limits, with at least one acceptable value at each concentration.

Assay quality control samples were prepared at three concentrations: approximately 15% above the LLOQ, approximately 15% below the upper limit of quantitation, and near the middle of the analytical range for each analyte. All assay quality control samples were used to measure assay precision, other than those rejected for analytical reasons. The data for each analyte were analyzed by one-way analysis of variance (ANOVA) to give estimates of both intra- and inter-day variability of the assay, using the analytical run sequence as the grouping variable. For the urine assay validation to be considered acceptable, both intra- and inter-day precision were required to yield a relative standard deviation (RSD) of $\leq 15\%$ at all concentrations. The cerebrospinal fluid assay was conducted on a limited number of samples over only two analytical runs, so while intra-day precision was obtained and required to meet the $\leq 15\%$ threshold of acceptability, inter-day precision data were not obtained. In addition, a pipetting problem led to two of the mid-range cerebrospinal fluid assay quality control samples being dropped from the analysis.

Recovery of analytes was not determined for the assays, because no extraction, concentration, or derivatization was involved in sample processing. The samples were simply diluted and chromatographed.

The LLOQ for each analyte was determined as the concentration at which the criteria were met for accuracy, precision, and specificity, as described above. The upper limits of quantitation were set arbitrarily except for 361W, which was nearing the upper limits of linearity for detector response at a concentration of approximately 56 μg/ml.

2.9. Stability of analytes

The stability of urine assay quality control samples was determined for three freeze–thaw cycles, after heating at 58°C for 5 h in order to inactivate HIV-1 virus, when stored at approximately −20°C for 6 weeks, and in processed samples stored for 7 days in the autosampler at approximately 4°C. Samples that had gone through two freeze–thaw cycles and met

assay stability criteria were thawed a third time and assayed after being stored for a total of 6 weeks at approximately −20°C. After each storage period, six replicates each of stability samples and freshly prepared assay quality control samples were assayed and the results were compared. Results of these studies were required to meet the accuracy and precision criteria stated above. Cerebrospinal fluid samples were always assayed within a few days of being obtained from the subjects. The results of the urine stability studies described below, as well as previous studies demonstrating the stability of the analytes for up to 2 years of frozen storage in analytical stock solutions and in plasma from a variety of species (data not shown), indicate that 1592U, 2269W, and 361W do not have any inherent instability issues. Because of this known stability information, and because the cerebrospinal fluid matrix is devoid of enzyme activity and has very limited protein content, it was judged that stability studies were not necessary in cerebrospinal fluid.

3. Results and discussion

3.1. Assay specificity

Comparison of chromatograms of a pre-dose urine sample from a clinical trial subject (Fig. 2B), a urine sample from the same subject 4–8 h post-dose (Fig. 2C) and a calibration standard at the LLOQ (Fig. 2A) showed that a small chromatographic peak in study samples occasionally eluted with similar retention time to abacavir or one of the two metabolites. These peaks were rarely as large as 20% of the LLOQ peaks, and the mean of these peaks in an analytical run never was close to the 20% threshold. Analysis of data from multiple analytical sequences demonstrated that these occasional co-elutants did not preclude accurate and precise quantitation. Comparison of chromatograms of cerebrospinal fluid samples obtained pre-dose from a clinical trial subject (Fig. 3B) with a sample obtained 2.5 h postdose (Fig. 3C) and with a calibration standard at the LLOQ (Fig. 3A) demonstrates the excellent specificity and chromatographic resolution of the assay.

3.2. Accuracy, precision, and limits of quantitation

The accuracy and precision data for validation of the urine and cerebrospinal fluid assays are summarized in Tables 1 and 2, respectively. Quantitation of abacavir, 2269W, and 361W used the parameter estimates from a linear least-squares regression analysis of the chromatographic peak area on the spiked concentration of the calibration curve data for each analyte with a weighting factor of $1/x^2$. The usual calibration curve consisted of duplicate sets of five separate calibration standard dilutions. Results from the urine assays demonstrated minimal deviation from the nominal spiking concentrations of the quality control samples (mean bias ranged from −3.81 to 3.00, 2.60 to 5.09, and −3.81 to 2.31% for abacavir, 2269W, and 361W, respectively). Intra-day RSDs of the urine assay were 0.55–2.28, 0.75–2.18, and 0.77–2.27% for abacavir, 2269W, and 361W, respectively, and inter-day variability was also excellent, with RSDs ranging from 1.05 to 1.56, 0.88 to 2.29, and 0.86 to 2.01% for abacavir, 2269W, and 361W, respectively. The cerebrospinal fluid assays also demonstrated acceptable bias from the nominal concentrations (mean bias ranged from 1.15 to 6.65, 1.31 to 6.35, and 0.99 to 6.59% for abacavir, 2269W, and 361W, respectively) and good precision, with RSDs of 1.15–6.65, 1.31–6.35, and 0.99–6.59% for abacavir, 2269W, and 361W, respectively.

The validated calibration curve ranges for abacavir, 2269W, and 361W were from 0.629 to 56.6, 0.296 to 26.4, and 0.579 to 52.1 μg/ml, respectively, for the urine assay. The validated calibration curve ranges for abacavir, 2269W, and 361W in the cerebrospinal fluid assay were from 0.062 to 5.13, 0.061 to 5.10, and 0.062 to 5.13 μg/ml, respectively. Correlation coefficients (r^2) of calibration curves were >0.99 as determined by least-squares analysis.

3.3. Stability of analytes

The stability of abacavir, 2269W, and 361W under various storage conditions is shown in Tables 3–5. All three analytes met the described stability criteria under all tested conditions. The mean bias in urine frozen at approximately −20°C for 6 weeks inter-

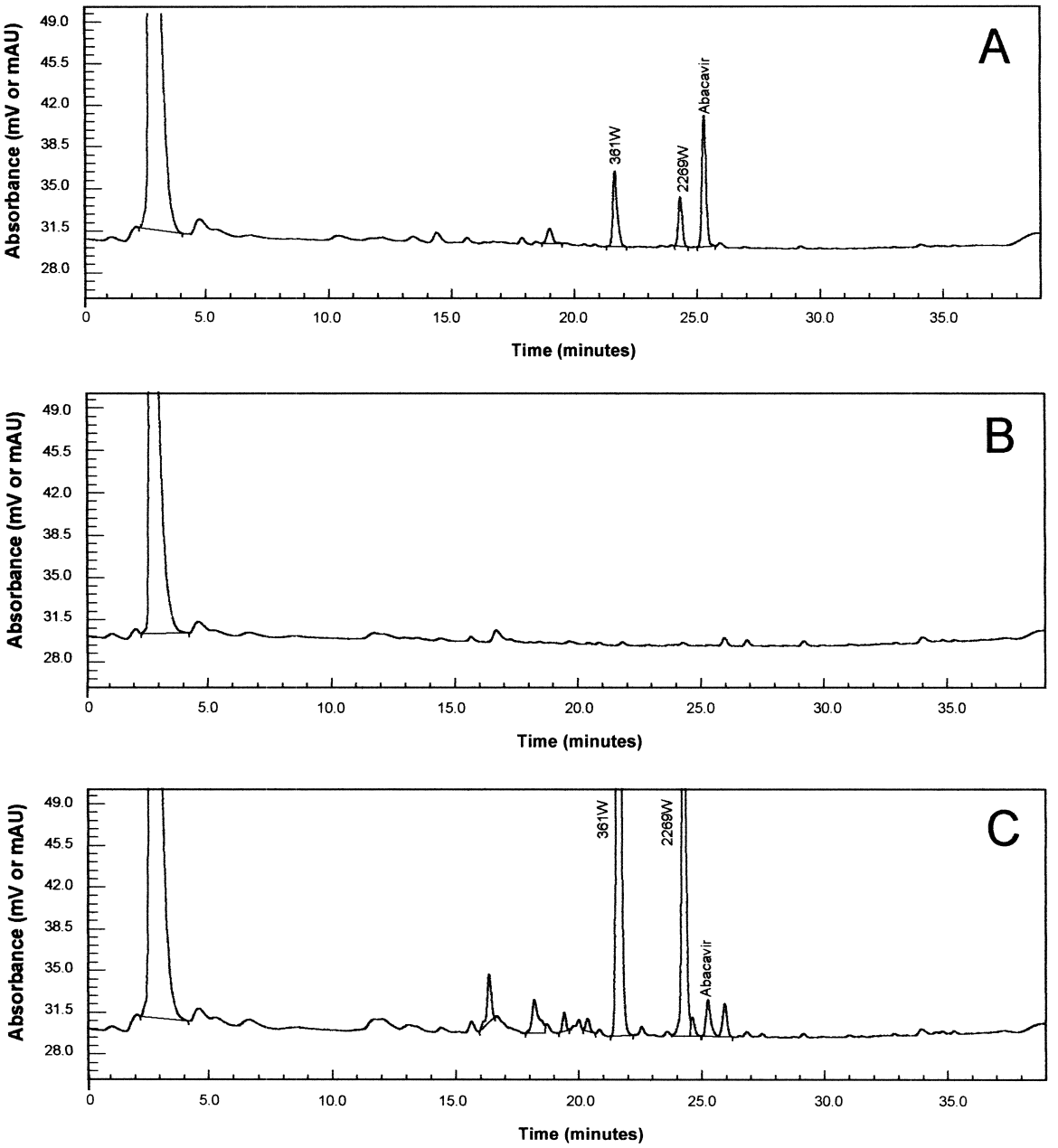

Fig. 2. Chromatograms from a calibration standard at the LLOQ (A), a pre-dose urine sample (B), and a urine sample collected from the same subject 4–8 h after dosing (C).

rupted by three freeze–thaw cycles ranged from −3.41 to 5.75%, and the precision was always within 3.44% of nominal, while the bias after storage of diluted urine samples for 7 days at approximately 4°C in the autosampler ranged from −9.44 to 11.7%. The greater than typical bias observed in many of the

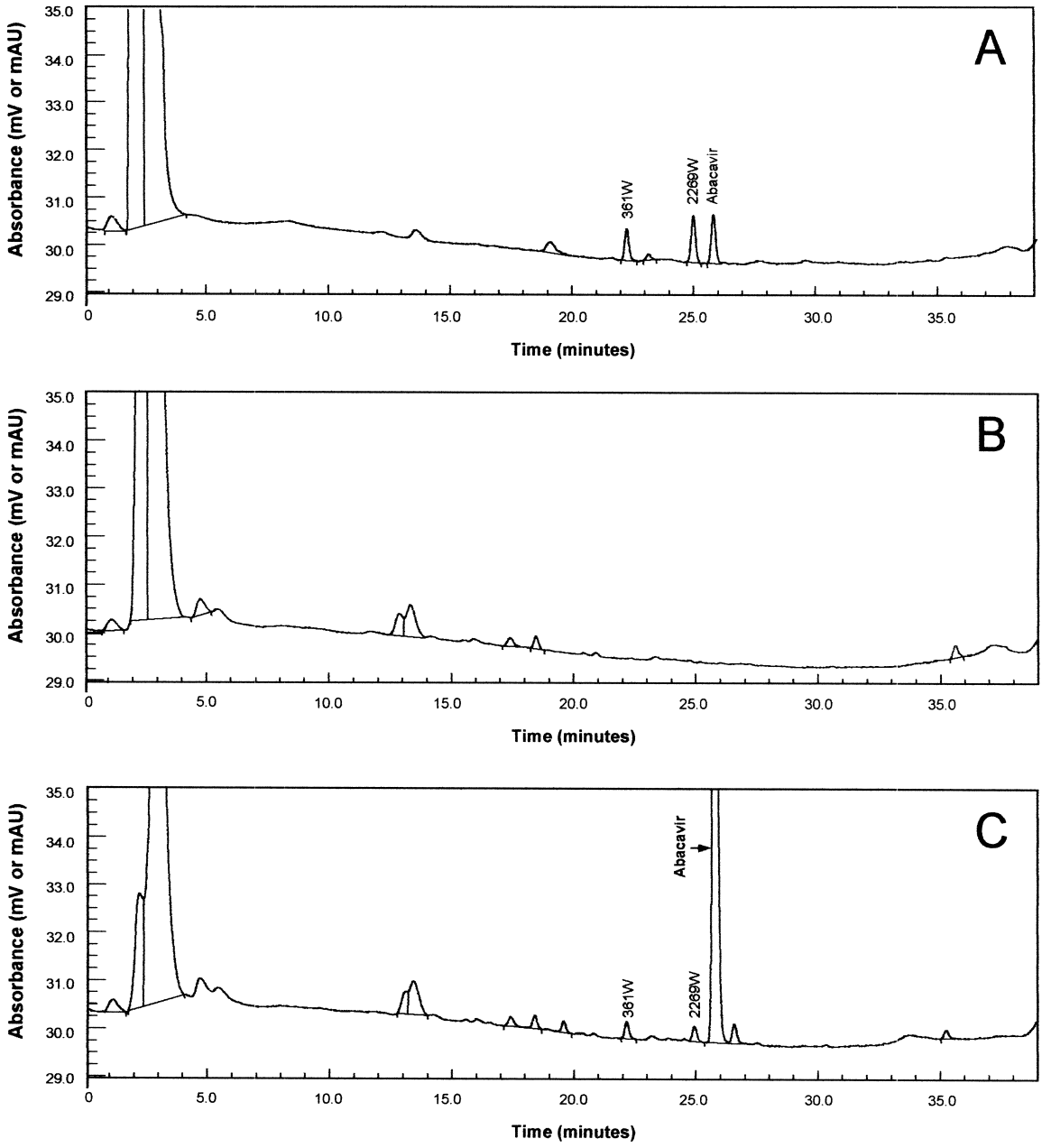

Fig. 3. Chromatograms from a calibration standard at the LLOQ (A), a pre-dose cerebrospinal fluid sample (B), and a cerebrospinal fluid sample collected from the same subject 2.5 h after dosing (C).

assays of samples stored for 7 days in the refrigerated autosampler is likely attributable to condensation or evaporation, due to the use of injection vials that were not self-sealing. After this experiment, vials that might need to be re-injected were routinely resealed immediately after assay, and the bias

Table 3
Stability of abacavir, 2269W, and 361W in human urine following heating to 58°C for 5 h

Spiked concentration (μg/ml)			Precision[a] (RSD, %)			Bias[a] (%)		
361W	2269W	Abacavir	361W	2269W	Abacavir	361W	2269W	Abacavir
1.74	0.888	1.89	2.77	2.58	3.63	3.45	0.45	2.12

[a] $n=6$/group.

Table 4
Stability of abacavir, 2269W, and 361W in human urine following three freeze–thaw cycles and storage at approximately −20°C for 6 weeks

Spiked concentration (μg/ml)			Precision[a] (RSD, %)			Bias[a] (%)		
361W	2269W	Abacavir	361W	2269W	Abacavir	361W	2269W	Abacavir
0.723	0.336	0.787	2.61	3.44	2.88	−3.41	5.75	−2.22
44.9	22.7	48.8	0.88	0.78	0.88	−2.26	−1.03	−0.65

[a] $n=6$/group.

became much tighter and more consistent both within and between sets of injections.

3.4. Analysis of clinical samples

The described assays for abacavir, 2269W, and 361W in human urine and cerebrospinal fluid have been utilized in a variety of distribution, drug interaction and special population studies of abacavir. Fig. 4 shows the individual cerebrospinal fluid concentration–time profiles of abacavir from subjects enrolled in a single dose, oral distribution and metabolism study of abacavir [7]. Of interest is that concentrations of abacavir in cerebrospinal fluid were found to be well above the in vitro IC_{50} (0.07 μg/ml) for at least 6 h, and that virtually no 2269W and 361W were detectable. Urine assays from six subjects enrolled in this study demonstrated that the 2269W and 361W each accounted for approximately 30–36% of the 600 mg dose, while parent abacavir only accounted for approximately 1% of the dose [7].

4. Conclusions

A simple, reversed-phase HPLC assay was developed and validated for quantitation of abacavir and its two major metabolites in urine and cerebrospinal fluid. The method met Glaxo Wellcome validation criteria for specificity, accuracy, and precision. The analytes were demonstrated to be stable under a variety of storage conditions. The utility of the assay in studying pharmacokinetic characteristics of clinical samples has been demonstrated.

Table 5
Stability of abacavir, 2269W, and 361W in diluted human urine stored in an HPLC autosampler at approximately 4°C for 7 days between injections

Spiked concentration (μg/ml)			Precision[A] (RSD, %)			Bias[A] (%)		
361W	2269W	Abacavir	361W	2269W	Abacavir	361W	2269W	Abacavir
0.723	0.336	0.787	0.63[B]	0.44[B]	0.41[B]	−8.53[B]	3.17[B]	−9.44[B]
8.68	4.40	9.44	4.34	3.90	4.01	1.42	4.43	0.72
44.9	22.7	48.8	0.73	0.78	0.67	8.54	11.7	8.33

A=($n=6$/group, except as noted), B=($n=3$/group). Note: Bias is reported as mean percent difference from nominal concentration.

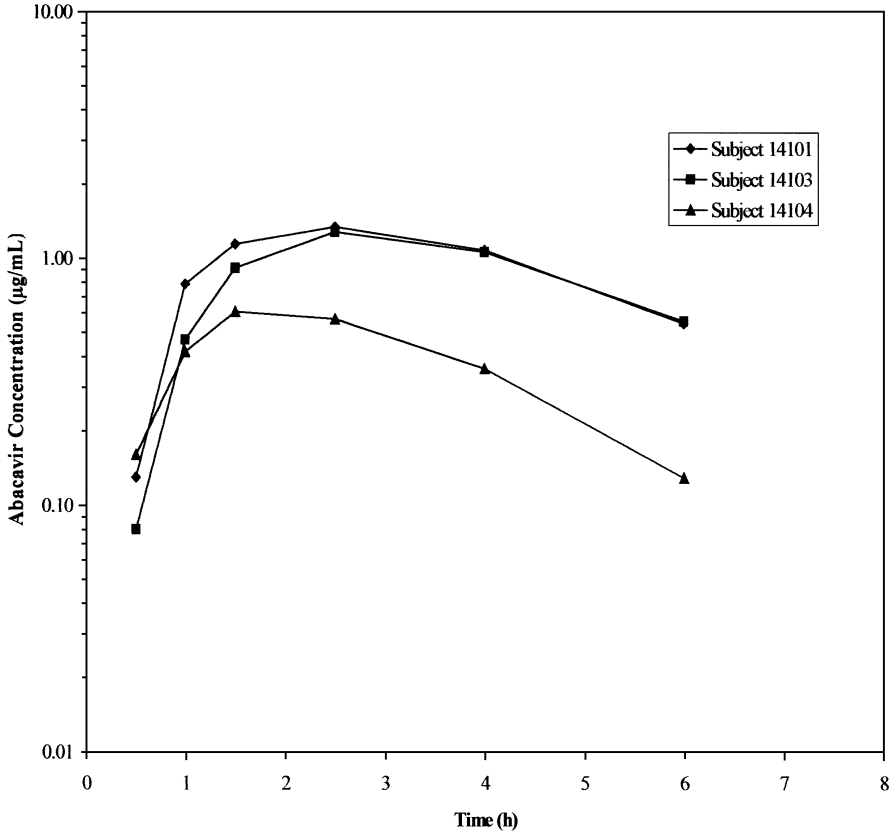

Fig. 4. Cerebrospinal fluid concentration versus time profiles for abacavir in three subjects from an abacavir oral distribution and metabolism study.

Acknowledgements

We would like to thank Kenneth R. Brouwer, David J. Reynolds and Steven S. Good for their support and technical advice, and James P. McDowell for providing the pharmacokinetic data.

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