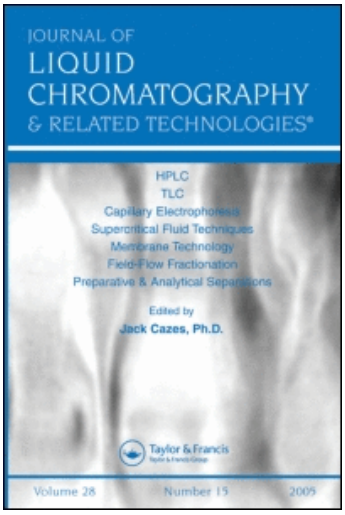

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Simple and Reliable HPLC Method of Abacavir Determination in Pharmaceuticals, Human Serum and Drug Dissolution Studies from Tablets

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Abstract: This work describes a new, fully validated, simple, rapid, selective, and sensitive HPLC method with UV detection for the direct determination of abacavir in pharmaceutical dosage forms, raw materials, spiked human serum, and drug dissolution studies without any time-consuming extraction or evaporation steps prior to drug assay. The mobile phase employed was methanol : acetonitrile: 0.015 M KH_2PO_4 (36 : 2.6 : 61.4 v/v/v) adjusted to pH 6.9 with 5 N NaOH. The samples of 20 μL were injected onto a Waters Spherisorb ODSI (250 × 4.6 mm, 5 μm particle size) column. Ketoprofen was used as internal standard. The flow rate was 1.0 mL min^{-1}. The retention times were 5.49 min for abacavir and 9.15 min for ketoprofen in mobile phase, 5.46 min for abacavir and 9.24 min for ketoprofen in serum samples. The samples were detected at 284 nm. The assay was linear in the concentration range 0.010–20 μgmL^{-1} ($r = 0.999$) with a slope of 1.35×10^{-3}; intercept of 0.0841 and the limit of detection was 0.00093 μg mL^{-1} in mobile phase and 0.025–20 μg mL^{-1} ($r = 0.999$) with a slope of 1.44×10^{-3}; intercept of 0.0733 and the limit of detection was 0.00418 μg mL^{-1} in human serum. The linearity of the detector response for abacavir was determined by plotting peak area ratios vs. concentration. It was successfully applied to the analysis of abacavir pharmaceutical

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preparations, and human serum samples without any interference by the excipients and endogenous substances. Moreover, the method can be used for the determination of abacavir for monitoring its concentration for in vitro dissolution studies.

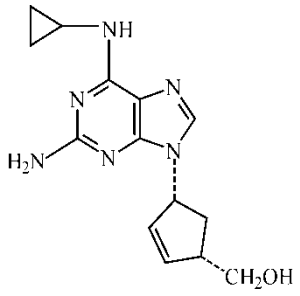
Keywords: Abacavir determination, drug dissolution study, human serum, pharmaceutical dosage form

INTRODUCTION

Abacavir {((1*S*, *cis*)-4-(2-amino-6-(cyclopropylamino)- 9*H*-purin-9-yl] cyclopent-2-enyl) methanol} is an anti-HIV drug that reduces the amount of virus in the body (Scheme 1). Anti-HIV drugs, such as abacavir, slow down or prevent damage to the immune system and reduce the risk of developing AIDS-related illnesses. Intracellularly, abacavir is converted by cellular enzymes to the active metabolite, carbovir triphosphate. Carbovir triphosphate is an analogue of deoxyguanosine-5′ triphosphate. Carbovir triphosphate inhibits the activity of HIV-1 reverse transcriptase both by competing with the natural substrate deoxyguanosine-5′ triphosphate and its incorporation into viral DNA.[1,2] Abacavir is one of the nucleoside analogues or reverse transcriptase inhibitors (NRTIs). These drugs block an HIV protein or enzyme called reverse transcriptase, which is involved in making new viruses.

The drug is safe and well tolerated in both children and adults. Abacavir is rapidly absorbed following oral administration with a bioavailability of about 80%. It is about 50% bound to plasma proteins. The elimination half-life is about 1.5 hr following a single dose (PDR 2002; Sweetman 2002). The adult dose is the equivalent of 300 mg of abacavir twice daily. Abacavir is metabolized into the pharmacological inactive 5′-glucuronide and the 5′-carboxylate.

Few procedures have been described for abacavir determination by liquid chromatography.[3–7] The reported methods were influenced by interference



Scheme 1. Structure of abacavir.

of endogenous substances and potential loss of drugs in the re-extraction procedure and involving lengthy, tedious, and time-consuming plasma sample preparation and extraction processes, and requiring a sophisticated and expensive instrumentation.

In the open literature, there appears to be no analytical method for the determination of abacavir, either in bulk form or in pharmaceutical dosage forms that have been reported up to date, except one voltammetric study,[8] The dissolution rate studies and related kinetic parameters of abacavir from tablets of this compound also have not yet been reported.

The determination of dissolution profiles for solid dosage forms of pharmaceuticals is required by pharmaceutical factories and research institutes. Drug dissolution testing is an integral part of pharmaceutical development and routine quality control monitoring of drug release characteristics. It is performed under precisely specified conditions of temperature, volume, and stirring rate, which may mimic processes in the human gastro-intestinal tract. The in vitro dissolution profiles obtained from dissolution rate studies have also been used for the successful characterization of the in vivo behavior of drugs.[9–13]

HPLC methods are useful in the determination of drugs in pharmaceutical dosage forms and biological samples. Owing to the widespread use of HPLC in routine analysis, it is important that good HPLC methods are developed and that these are thoroughly validated.[14–17]

The goal of this work was the development of a new fully validated, simple, rapid, selective, and sensitive HPLC method with UV detection for the direct determination of abacavir in pharmaceutical dosage forms, raw materials, spiked human serum, and drug dissolution studies, without any time-consuming extraction or evaporation steps prior to drug assay.

EXPERIMENTAL

Apparatus

An HP chromatographic system (Hewlett Packard, Avondale, USA) consisting of a Model Agilent 1100 series with a Model Agilent series G-13158 DAD detector and a Model Agilent 1100 series G-1329 ALS auto sampler were used. The chromatograms were analyzed with Agilent Technologies HPLC 1100 software. The separation was carried out at ambient temperature, on a reversed-phase Waters Spherisorb ODSI column (250 × 4.6 mm, 5 μm particle size). The chromatographic separation was performed using an isocractic mode. The mobile phase consisted of a mixture of methanol : acetonitrile: 0.015 M KH_2PO_4 (36 : 2.6 : 61.4 v/v/v) adjusted to pH 6.9 with 5 N NaOH and delivered at a flow rate of 1.0 mL min^{-1}. The UV detector was

set at a wavelength of 284 nm. An injection volume of 20 μL was used. Ketoprofen was used as an internal standard.

The dissolution rate studies of VAL from tablets were performed on Caleva 7ST dissolution apparatus (G.B. Caleva Inc., England).

Standard Solutions and Calibration Curves

The standard abacavir and internal standard ketoprofen (IS) were prepared by dissolving 10 mg of compounds with 10 mL of mobile phase in a 10 mL volumetric flask. The concentration of abacavir was varied in the range of $0.010-20.00\,\mu g\,mL^{-1}$ and the concentration of IS was maintained at a constant level of $1.0\,\mu g\,mL^{-1}$. The calibration curve for HPLC analysis was constructed by plotting the ratio of the peak area of the drug to that of internal standard against the drug concentration.

Ruggedness, Precision, and Accuracy

The ruggedness and intra-day and inter-day precision and accuracy of the method were estimated by assaying five replicate samples at three different concentrations, on the same day and on five different days over a 2 weeks period, respectively. The relative standard deviations (RSDs) were calculated to check the ruggedness and precision of the method. The accuracy was expressed as percentage bias.[18,19]

$$\text{Bias} = \frac{(\text{Nominal concentration} - \text{Measured concentration})}{\text{Nominal concentration}} \times 100$$

Analysis of Tablets

Ten tablets were weighed, crushed, and combined. An amount of powder equivalent to about 10 mg abacavir was accurately weighed, transferred into a 10-mL volumetric flask, diluted with mobile phase, sonicated for 10 min, and then completed to volume with the same solution. After filtration, appropriate solutions were prepared by taking suitable aliquots of clear filtrate and adding the appropriate IS solution, diluting them with mobile phase in order to obtain a final solution.

Recovery Studies

To keep an additional check on the accuracy of this developed assay method, recovery experiments were performed by adding the known amount of pure drug to pre-analyzed samples of tablets. The percent recovery was calculated by comparing the concentration obtained from spiked samples with actual added concentration. Thus, the effect of common tablet formulation excipients on chromatograms (e.g., tail, broadening, etc.) was investigated. Recovery experiments also showed the reliability and suitability of the proposed method. The known amounts of the pure drug and internal standard at a constant level were added to abacavir pharmaceutical formulation and the mixtures were analyzed by the proposed method. After five repeated experiments the recoveries were calculated.

Recovery Studies in Spiked Human Serum Samples

Drug free plasma samples, obtained from healthy individuals (after obtaining their written consent), were stored frozen until assayed. After gentle thawing, 1 mL aliquots of serum samples were spiked with 100 μg mL^{-1} of abacavir (dissolved in mobile phase), 1 mL methanol : acetonitrile (1 : 1 v/v for denaturation and precipitation of plasma proteins). The tubes were tightly capped and vortex-mixed for 10 min and then centrifuged for 10 min at 5000*g*. The supernatant was taken carefully. The concentration of abacavir was varied in the range of 0.025–20 μg mL^{-1} in human serum samples, and the concentration of IS was maintained at a constant level of 1 μg mL^{-1}. Serum samples were injected to the column. Standard curves were constructed for abacavir in serum using the ratio of the observed peak area for abacavir to the IS. Unknown concentrations were computed from the linear regression equation of the peak area ratio against the concentration of abacavir.

In Vitro Dissolution Studies

Drug dissolution studies were carried out according to the USP 24[20] dissolution procedure for the single entity products, with use of a paddle-stirrer type of apparatus in 900 mL of 0.1 M HCl (pH 1.2, gastric medium), at a stirring rate of 75 rpm. The temperature of the cell was maintained at 37 $\pm$ 0.5°C by use of a thermostatic bath. At each sample time interval, an exact volume of sample was withdrawn from each flask and immediately replaced with an identical volume of fresh medium. At predetermined time intervals (0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 10, 20, 30, 45, and 60 min) the concentrations of abacavir in the dissolution medium were determined from a linear regression equation of the proposed method. The cumulative percentage drug released in

media was plotted against time in order to determine the release profile of the drug from each formulation.

RESULTS AND DISCUSSION

To develop a rugged and suitable HPLC method for the quantitative determination of abacavir, various mobile phase compositions and ratios were employed. Our preliminary trials using different compositions of mobile phases consisting of water, methanol, and acetonitrile, and different ratios of this solution, did not give good peak shape. Addition of KH_2PO_4 buffer (0.015 M) instead of water improved the peak shape of abacavir. Finally, by fixing buffer at pH 6.9 and mobile phase composition consisting of a mixture of methanol : acetonitrile : 0.015 M KH_2PO_4 (36 : 2.6 : 61.4 v/v/v), abacavir and IS were resolved to the baseline and obtained the best peak shape. This mobile phase composition was found to be optimal for good peak shape as well as to achieve minimal background current.

Precision and accuracy can often be enhanced by the use of an appropriate internal standard for an HPLC method, which also serves to correct for fluctuations in the detector response. One of the main reasons for using an internal standard is for samples requiring significant pretreatment or preparation. Often, a sample preparation step that include reaction, filtration, precipitation, extraction, and so on, results in sample losses. When added prior to sample preparation, a properly chosen internal standard can be used to correct for these sample losses. The internal standard is a different compound from the analyte, but one that is well resolved in the separation. The chemical structure of ketoprofen is not similar to the abacavir structure. However, it was chosen as the internal standard because it showed a shorter retention time with better peak shape and better resolution, compared with other potential internal standards. Finally, using the conditions selected earlier, a satisfactory chromatographic peak resolution was obtained in a short analysis time, as can be seen in Figure 1. For both compounds, sharp and symmetrical single peaks were obtained with good resolution.

System suitability tests are an integral part of HPLC method development.[20] It can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. The criteria selected will be based on the actual performance of the method, as determined during its validation. System suitability was checked by evaluating different parameters (retention time, tailing factor, capacity factor, resolution, and selectivity). Typically, at least two of these criteria are required to demonstrate that system suitability tests were carried out on freshly prepared standard stock solutions of abacavir. Tailing and capacity factors were obtained as 1.36 and 2.40 for abacavir and 1.13

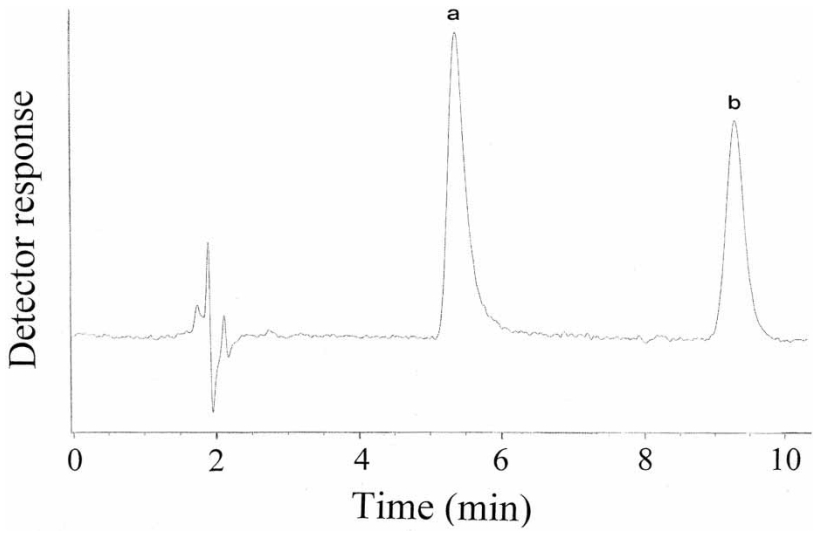


Figure 1. Chromatogram obtained from tablet dosage form containing 1.00 μg mL^{-1} abacavir and 1.00 μg mL^{-1} ketoprofen (IS).

and 4.34 for IS, respectively. The presented chromatographic conditions ensure adequate retention of both compounds, since capacity factor values obtained satisfied the conditions ($\geq$1.0). Resolution and selectivity factors for this system were found to be 5.02 and 2.003, respectively. The method has enabled good resolution of analytes, since values of resolution factors of adjacent peaks were greater than 1.0. The retention times in mobile phase were used as hold-up time for the capacity factor calculations. The retention times of abacavir standard sample, tablets, and serum samples were 5.49, 5.37, and 5.46 min, respectively. The retention times of ketoprofen were obtained as 9.15 min in mobile phase, 9.30 min in tablets, and 9.24 min in serum samples. The variation in retention time among five replicate injections of abacavir reference solution was very little in raw material, tablets, and serum samples, giving RSD% of 0.058%, 0.243%, and 0.064%, respectively. The results obtained from system suitability tests are in agreement with the USP requirements.

The calibration curves for abacavir in mobile phase and serum samples were drawn by plotting the peak area ratio of abacavir to IS, vs. concentration of abacavir, to yield the correlation coefficient (r) of 0.999 in both media over the concentration range 0.010–20.00 μg mL^{-1} and 0.025–20.00 μg mL^{-1}, respectively. Values obtained for the calibration curve and their related validation parameters are presented in Table 1. The low values of SE of slope and intercept, and greater than 0.999 correlation coefficient in both media, established the precision of the proposed method. Several approaches are given in

Table 1. Characteristics of the linear regression analysis of abacavir

	Mobile phase	Human serum
Linearity range ($\mu g\,mL^{-1}$)	0.010–20.00	0.025–20.00
Slope	1.35×10^{-3}	1.44×10^{-3}
Intercept	0.0841	0.0733
Correlation coefficient (r)	0.999	0.999
SE of slope	1.17×10^{-5}	5.56×10^{-6}
SE of intercept	0.0814	0.041
Detection limit ($\mu g\,mL^{-1}$)	0.00093	0.00418
Quantification limit ($\mu g\,mL^{-1}$)	0.00309	0.0139

the ICH guideline to determine the detection (LOD) and quantitation (LOQ) limits. In this study, LOD and LOQ were based on the standard deviation of the response and the slope of the corresponding calibration curve, using the following equations: $LOD = 3\,s/m$; $LOQ = 10\,s/m$.

The stability of the reference substance and sample solutions were checked by analyzing a prepared standard solution of abacavir in mobile phase aged at $+4^{\circ}C$, in the dark against a sample freshly prepared. The results demonstrated that the working reference solutions were stable for up to 2 weeks. The abacavir area ratio to IS for the assay reference solutions over 2 weeks did not change considerably.

The developed method was validated according to the standard procedures,[18,19] and the results obtained are tabulated in Table 2. Accuracy, precision, and reproducibility of the proposed method were assessed by performing replicate analysis of the standard solutions in mobile phase and serum. Within the calibration curves, two different concentrations were prepared in both media and assayed with related calibration curves to determine within-day and between-day variability. The within-day and between-day precision, accuracy, and reproducibility were determined as the RSD% and mean value and the results were shown in Table 2. Precision, accuracy, and reproducibility results shown in Table 2 demonstrate good precision, accuracy, and reproducibility.

When working on standard solutions, and according to the obtained validation parameters, results encourage the use of the proposed method described for the assay of abacavir in pharmaceutical dosage forms, spiked human serum samples, and drug dissolution studies.

The results obtained from the analysis of tablet dosage form are summarized in Table 3. Recovery experiments were realized by using the standard addition method. Recovery experiments using the developed assay procedure further indicated the absence of interference from commonly encountered pharmaceutical excipients used in the selected formulation (Table 3).

Table 2. Intra-day and inter-day precision of abacavir standards

Theoretical concentration ($\mu g\,mL^{-1}$)	Mobile phase				Serum samples			
	Within-day measured concentration ($\mu g\,mL^{-1}$)[a]		Between-day measured concentration ($\mu g\,mL^{-1}$)		Within-day measured concentration ($\mu g\,mL^{-1}$)[a]		Between-day measured concentration ($\mu g\,mL^{-1}$)	
	Mean	RSD%	Mean	RSD%	Mean	RSD%	Mean	RSD%
100	101.22	1.12	99.92	1.69	101.24	1.22	101.65	1.64
1000	992.61	0.82	997.78	1.31	993.05	0.81	1000.21	1.61

Note: Between-day reproducibility was determined from three different runs over a 2 weeks period.
[a]Mean values represent three different abacavir standards for each concentration.

Table 3. Results of the assay and the recovery analysis of abacavir in tablets and spiked human serum samples

	Tablets (mg)	Serum samples ($\mu g\,mL^{-1}$)
Labeled claim (mg per tablets)	300	
Mean of amount found[a]	296.37	
RSD% of amount found	1.25	
Bias%	1.21	
Added	50.0	2.50
Recovered[a]	50.07	2.45
Recovery%	100.15	98.11
Bias%	−0.14	1.89
RSD% of recovery	0.59	0.94

[a]Each value is the mean of five experiments.

In order to check the applicability of the proposed method to the human serum samples, the calibration equation was obtained in spiked serum samples. Recovery studies were also performed in human serum samples and the results were calculated by using the related calibration equation. Calibration equation parameters were shown in Table 1. Related validation parameters and obtained recovery results of spiked serum samples were given in Tables 2 and 3, respectively. Analysis of drugs from serum samples by HPLC usually requires extensive time-consuming sample preparation, use of expensive organic solvents, and other chemicals.[21,22] In our proposed method, the serum proteins are precipitated by the addition of methanol, which is centrifuged at 5000*g*, and the supernatant is diluted, directly injected, and analyzed. Figure 2 shows the typical chromatogram obtained, the blank serum (a) and serum spiked with abacavir and constant amount IS (b). As can be seen in Figure 2, there are no extraneous peaks in chromatograms obtained for serum samples. The determined results and recoveries of known amounts of abacavir added to serum samples are given in Table 3. The proposed method gives reproducible results, is easy to perform, and is sensitive enough for the determination of abacavir in human serum samples.

The proposed method was also applied to the determination of abacavir in dissolution rate studies of the samples obtained from the tablets. Abacavir tablet formulation, which includes 300 mg active compound, was investigated with the paddle dissolution method. It is essential to consider the in vitro dissolution tests as important criteria for the quality of the marketed dosage forms if obtained from various sources, and it can judge the suitability of this formulation to deliver the required active substance properly to the patient. Thus, the need of dissolution tests of drug formulations is

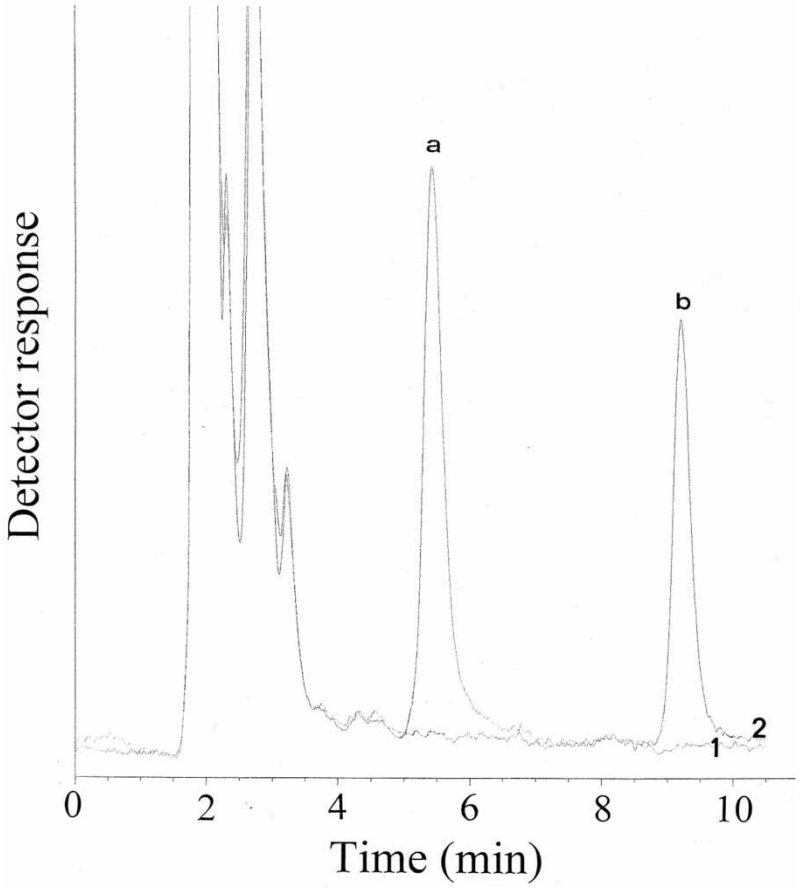


Figure 2. Chromatogram of blank serum (a) and serum spiked with 1.00 μg mL^{-1} abacavir and 1.00 μg mL^{-1} ketoprofen (IS) (b).

indispensable to ensure good drug quality. Hence, the method can be easily and conveniently adopted for routine quality control analysis of abacavir. The cumulative percentage drug released in dissolution media vs. time profile is shown in Figure 3. As can be seen in Figure 3, more than 90% drug dissolved in this media within 2.5 min. The release data were evaluated according to the different models namely zero order, first order, Hixson-Crowell, Weibull distribution (RRSBW)[23,24] function, and Peppas equation.[25,26] Shown in Table 4 are all the kinetics, related rate constants, and parameters. For the releasing profile (Figure 3), best compliance according to the highest determination coefficient and lowest AKAIKEs information criteria and SWSD value for abacavir dosage form was found

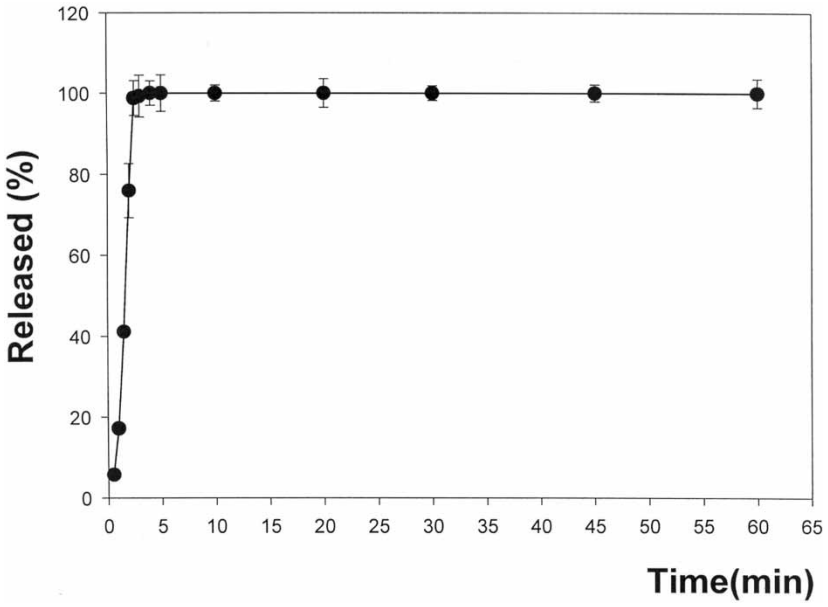


Figure 3. In vitro dissolution profiles of abacavir tablets by proposed RP-HPLC method.

to be Weibull distribution. The release of abacavir from tablets was determined to be 63.2%, at the end of 1.00 min. Shape factor (β) value was found to be lower than 1.0. The release of abacavir from the tablets tested were completed within 3.0 min in the proposed method. In order to understand the magnitude of the diffusional exponent n of drug from tablets, dissolution data were fitted to the Peppas equation where n is a factor, which indicates the mechanism of the release; for instance, $n : 0.5$ for square root of time and $n : 1$ for zero order release. The exponent value was found to be 0.408 (Table 4). According to the Peppas equation, the results were in agreement with $Q\sqrt{t}$ kinetics.

CONCLUSION

Using the described chromatographic conditions, abacavir and IS were well separated. The HPLC method developed in this study has the advantage of simplicity, precision, and reliability. It allows for the direct determination of abacavir, bypassing several tedious steps. The proposed method gives good resolution between abacavir and internal standard within a short

Table 4. Kinetic parameters of release data of abacavir tablets

Zero order	kr°	138.66
	r^2	0.183
	SWSD	5.21
	AKAIKEs information criteria	25.137
First order	kr	4.907
	r^2	0.351
	SWSD	0.424
	AKAIKEs information criteria	15.904
Hixson-Crowell	k	6.005
	r^2	0.268
	SWSD	4.071
	AKAIKEs information criteria	16.309
Weibull distribution (RRSBW)	$T_{(\min)}$	1.00
	β	0.749
	r^2	0.642
	SWSD	23.98
	AKAIKEs information criteria	−4.871
Peppas Equation	k_p	0.107
	n	0.408
	r^2	0.481

Note: kr: Release rate constant of first order kinetic; kr°: Release rate constant of zero order kinetic; k: Release rate constant of Hixson-Crowell kinetic; k_p: Release constant of Peppas equation, r^2: determination coefficient; SWSD: Sum of weighed squared deviations; β: shape factor; $T_{(\min)}$: value stands for the time for 63.2% release of the drug, n: diffusional exponent.

analysis time (<10 min). There was no significant difference for the assay tested within day and between days. The proposed method was applied to the direct determination of abacavir in tablet formulation. In order to check for the accuracy and precision of the developed method, the recovery studies were performed from the tablets. High percentage of recovery shows that the method is free from the interferences of the commonly used excipients and additives in the formulations of the drug. In order to check the applicability of the proposed method to the human serum samples, the recovery studies and linearity range studies were performed in human serum samples. There are no extraneous peaks in chromatograms obtained for serum samples. The proposed HPLC method was also applied to the determination of abacavir in dissolution rate studies of the samples obtained from tablets.

For the releasing profile, the best compliance according to the highest determination coefficient and lowest AKAIKEs information criteria for abacavir dosage form, was found to be Weibull distribution.

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