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ANALYSIS

DETERMINATION OF ACYCLOVIR IN RABBIT PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) TECHNIQUE

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Abstract: A rapid, sensitive and simple reversed-phase high-performance liquid chromatographic (HPLC) method has been developed and validated for the determination of acyclovir (ACV) in rabbit plasma. BDS C18 column was used to conduct analysis using ammonium dihydrogen phosphate buffer (50 mM) and methanol as mobile phase (98 : 2), with pH adjusted to 2.5 using orthophosphoric acid. The flow rate was kept at 1 mL/min. Selective precipitation of plasma proteins was done by adding 5% perchloric acid. Precipitated plasma proteins were separated by centrifugation. ACV moves in a supernatant, which was snapped and passed through a syringe filtration assembly. Direct injection of supernatant was given into a BDS C18 column and ACV was detected at 256 nm. The limit of detection for ACV in plasma was estimated as 15 ng/mL whereas the limit of quantitation was calculated as 25 ng/mL. Moreover, the developed method has been found to be selective and linear into concentration range of 25–2000 ng/mL. The present method could be successfully applied to samples from bioavailability and bioequivalence studies.

Keywords: reversed-phase high-performance liquid chromatography, acyclovir, plasma

A rise in the global prevalence of herpes virus infections have affected about more than the one-third population in the world. Generally, herpes simplex virus is regarded as a primary source of oral and genital infections in individuals infected with HIV and who are transplant recipients. These individuals are regarded as immune compromised patients and have 90% risks of having severe complications from HSV infections. ACV is regarded as one of the most effective and selective antiviral drugs used for prophylaxis and treatment against herpes simplex and varicella-zoster viruses (1). Antiviral activity of ACV is effective by competitive inhibition of viral DNA, through selective binding of ACV to HSV-thymidine kinase (2). From the gastrointestinal tract, ACV is partially absorbed after oral administration having bioavailability between 15% and 30%. 15% of the drug bounds to plasma protein whereas excretion of the major part of the drug occurs unchanged by the kidney (3). An average plasma half-life of ACV in adults with normal renal functioning is 1.5 h to 3 h.

One of the most important challenges in the estimation of ACV in biological samples is the structural similarity of ACV to endogenous agents. Hence, a highly selective analytical method is needed to be developed and validated for the estimation of ACV. Radioimmunoassays and enzyme-linked immunoabsorbent assays (ELISA) are regarded as highly sensitive techniques (4). However, their cost, time requiring experimental procedures and requirements of monoclonal antibodies are problematic and difficult. Moreover, manipulation and disposal of radioisotopes in radioimmunoassay make this method disadvantageous and inconvenient (5). Therefore, as an alternate, reversed-phase HPLC technique has been largely investigated for the estimation of ACV in human plasma using either UV (6-14) or fluorescence detection method (15-18). UV detection of the typical chromatographic method was done at 250-256 nm. Furthermore, using excitation wavelengths of 260-285 nm and emission wavelengths of 375-380 nm fluorescence detection was also conducted (19).

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In present work, we report a simple but highly efficient and reproducible method for the detection of ACV in plasma using HPLC-UV method. This method offers an advantage as it eliminated complex and lengthy extraction procedure, thus improving the overall speed of analysis and saving both time and money.

MATERIAL AND METHODS

Materials

ACV was gifted for conducting research work by Brooks Pharmaceuticals (Pvt) Ltd. (Karachi, Pakistan). Methanol and orthophosphoric acid was purchased from Merck (Germany). Perchloric acid were obtained from Applichem (Germany). Ammonium dihydrogen phosphate was purchased from Sigma Aldrich (UK). Microfilters of 0.4 microns were purchased from Millipore (Merck, Germany). Rabbit plasma was obtained from animal facility centre of the Faculty of Pharmacy and alternative medicine, the Islamia University of Bahawalpur, Pakistan. All chemicals and reagents used were of HPLC grade.

Instrument and chromatographic condition

A Shimadzu HPLC system, Agilent 1100 series (Kyoto, Japan) consisting of LC-10 AT VP pump, Rheodyne manual injector with a 20 µL loop, SPD-10 AVP UV–VIS detector and a DGU-14A degasser was used for setting the reverse-phase liquid chromatographic conditions. The separation of ACV was achieved using BDS Hypersil C18 column, 250 × 4.6 mm which was kept at 25 ± 2°C temperature. The chromatographic system is integrated via Shimadzu model CBM-102 communication bus module to P-IV computer loaded with CLASS-GC software (Version 5.03) for data acquisition and mathematical calculations. For isocratic elution, filtration was carried out for eluent through microfilters. Later, the eluent was degassed and delivered at the flow rate of 1 mL/min. The chromatographic run time was 10.0 min. The effluent was monitored with UV detection at 256 nm.

Preparation of the mobile phase was carried out by dissolution of measured quantity of ammonium dihydrogen phosphate i.e. 1.437 g in 250 mL of distilled water to obtain 50 mM buffer. Then mixing of prepared buffer was done with methanol in a ratio of 98 : 2, respectively. Further orthophosphoric acid (85%) was used to adjust pH to 2.5. After this, the prepared mobile phase was filtered through vacuum filtration assembly using 0.45 µm Millipore filter paper.

Standard stock and calibration standards

Preparation of stock solution of ACV i.e. 100 µg/mL took place by dissolving 10 mg of ACV in 100 mL of mobile phase used as a diluent. Moreover, preparations of serial dilutions were done from prepared stock solution in the range of 2000, 1000, 500, 300, 100, 50 and 25 ng/mL.

Sample preparation

Experimental protocol used in the present study was reviewed and approved by the Pharmacy Research Ethics Committee (PREC), the Islamia University of Bahawalpur, Pakistan (23-2016/PREC). For method development and validation, plasma samples were obtained from rabbits, kept at Animal facility centre of Faculty of Pharmacy and Alternative Medicine, the Islamia University of Bahawalpur, Pakistan. The blank plasma samples were then stored at -20°C. Before processing for analysis, frozen plasma samples were thawed and brought to room temperature.

For the preparation of calibration curve standards, 0.5 mL of blank plasma was spiked with 0.5 mL of ACV solution. Moreover, extraction of ACV was done from plasma by a simple and single step liquid-liquid extraction procedure. For this, the addition of 1 mL of 5% (v/v) freshly prepared perchloric acid was done to 1 mL aliquot of blank plasma or plasma spiked with a known concentration of ACV in a centrifuge tube. Mixing was done by vortexing for 10 min. Separation of precipitated plasma proteins was done by conducting centrifugation at 3500 rpm (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) for 15 min. As a result, ACV moves in a supernatant, which was snapped and passed through a syringe filtration assembly. Further, the injection of 20 µL of the sample prepared was given into the chromatographic system.

Method validation

Following the Food and Drug Administration (FDA) guidelines for Bioanalytical Method Validation, a thorough validation of the method was conducted to estimate ACV concentration in plasma (20). For this construction of plasma calibration curves were done to estimate specificity, linearity, precision and accuracy, recovery, limit of quantitation (LOQ), limit of detection (LOD) and stability.

Specificity

Both blank and spiked plasma samples were evaluated in terms of their selectivity. Generation of representative chromatograph was done to indicate that other analytes of samples matrix are separated from the parent components.

Standard curve and linearity

The calibration graph was established by plotting the mean peak area against ACV concentration. Linear least-square regression analysis was performed to assess the linearity and to generate the slope, y-intercept and correlation coefficient (r). The value of coefficient of correlation should be 0.9800 or greater.

Precision and accuracy

The validation of chromatographic method was carried out on a single (intra-day) analytical day and on different validation days (inter-day) to evaluate the precision and accuracy of the developed HPLC method. For this, intra-day variability of the method was evaluated by replicate analysis of five validation samples on the same day. Similarly, determination of inter-day variability was done by replicate analysis of five validation samples on five different days. Precision is measured as the percent of relative standard deviation (RSD, %) as mentioned in Equation 1. Accuracy was calculated as a percentage of relative error (R.E, %) as mentioned in Equation 2.

$$\text{Precision} = R.S.D = \frac{S.D}{\text{main concentration found}} \times 100 \quad (1)$$

$$\text{Accuracy} = \%\ R.E = \frac{\text{concentration found} - \text{concentration added}}{\text{concentration added}} \quad (2)$$

The mean precision and accuracy should be within by ±15.0% according to acceptance criteria. However, it can be ±20.0% of the nominal concentration for the lower limit of quantification (LLOQ).

Recovery

The extraction efficiency of the sample prepared by using 5% perchloric acid was evaluated at LQC, MQC and HQC levels. Comparison of mean area response from five replicates of extracted plasma samples (spiked before extraction) was done to that of un-extracted samples (spiked after extraction). Although the extraction efficiency is not required to be 100%, it should be reproducible and consistent.

Sensitivity

An analytical method has been tested in terms of sensitivity by establishing its lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). LLOQ is defined as the minimum concentrations of ACV which can be calculated and quantified. Besides, deviance between measured and nominal concentration at LLOQ must be within ±20%. LLOD is the concentration that can be detected but not quantitated.

Stability

To estimate the stability of prepared stock solution and plasma samples at low, medium and high-quality control levels (25 ng/mL, 300 ng/mL, and 2000 ng/mL), stability tests were conducted under various conditions. Evaluation of stability outcomes was done by estimating area response of stability samples against freshly prepared comparison standards having the same concentration. Stock solution of ACV was assessed for short term stability at 25^{o}C for 6 h and long term stability at 2-8^{o}C for 6 days. For plasma samples, room temperature or bench top stability at three QC levels was investigated up to 6 h. Evaluation of long term stability was done for 4 weeks at -20^{o}C for. Moreover, freeze-thaw stability was conducted in plasma by sequential cycles of freezing (at -20^{o}C for 24 h) and thawing at room temperature. The deviancy of plasma samples should be within ±15.0% when compared with the mean calculated concentration of freshly thawed samples. Stability studies (long term) after four weeks were not conducted due to no applicability (21). % change in concentration of stability and comparison samples have been calculated by following Equation 3.

$$\%\ Change = \frac{\text{Mean stability samples} - \text{Mean compasrision samples}}{\text{Mean compasrision samples}} \times 100 \quad (3)$$

RESULTS AND DISCUSSION

Optimization of method

Optimization of the method is a pre-requisite step for any chromatographic method. The purpose is to get analyte of interest with good resolution, sensitivity, reproducibility, precision, accuracy, recovery, and stability. Number of columns i.e. Hypersil BDS C 18 (250 × 4.6 mm), Hypersil ODS (250 × 4.6), Hypersil ODS (150 × 4.6), etc. with particle size 5 (µ) have been tested during method development to conduct separation of ACV. Moreover, investigation of different mobile phases was done either by altering the ratio of aqueous and organic components or by testing different buffers in various ratio with distilled water, acetonitrile, or methanol. Buffers tested include ammonium dihydrogen, phosphate, potassium dihydrogen phosphate, and sodium dihydrogen phosphate with the aim to attain an optimum separation of ACV in the pH range of 2-8 (9, 13, 22-24). It has been found that pH of higher range produced asymmetrical peaks. Therefore pH range of lower side 2.5-4 has been chosen for further investigations.

Among a number of columns tested, Hypersil BDS C18 (250 × 4.6) was finally chosen for HPLC

Table 1. Optimized Chromatographic conditions.

Chromatographic parameter	Optimized conditions
Flow rate	1 mL/min
Wavelength	256nm
Injection volume	20 µL
Retention time	6.3 min
Run time	10 min
Temperature	25°C
Pressure	108-116 bars
Mobile phase	Ammonium dihydrogen phosphate and Methanol 98 : 2
pH	2.5
HPLC column	BDS C18
HPLC detector	UV detector

Table 2. Calibration curves parameters of Acyclovir in spiked rabbit plasma.

Run number	Equation form Y = BX + A		Correlation coefficient
	B	A	(r^2)
Day 1	0.178	1.768	0.999
Day 2	0.174	2.582	0.999
Day 3	0.175	1.215	0.998
Mean ± SD	0.176 ± 0.002	1.855 ± 0.688	0.999 ± 0.0006

Table 3. Intra-day and inter-day precision and accuracy for determination of ACV in plasma.

Intra-day (n = 5)			
Parameters	LQC	MQC	HQC
	ng/mL	ng/mL	ng/mL
Nominal concentration	25	300	2000
Mean ± SD	23.30 ± 1.20	288.53 ± 6.65	1961.73 ± 23.60
Precision (% RSD)	5.16	2.3	1.2
Accuracy (% RE)	6.8	3.41	1.91
Inter-day (n = 5)			
Parameters	LQC	MQC	HQC
	ng/mL	ng/mL	ng/mL
Nominal concentration	25	300	2000
Mean ± SD	23.23 ± 1.02	288.10 ± 8.55	1963.23 ± 21.21
Precision (% RSD)	4.42	2.96	1.08
Accuracy (% RE)	7.06	3.96	1.83

Table 4. % Mean recovery (n = 5) of ACV in rabbit plasma.

Parameters	LQC	MQC	HQC
	ng/mL	ng/mL	ng/mL
Nominal concentration	25	300	2000
Peak area of spiked plasma (Before extraction)	21.08	275.29	1915.46
Peak area of spiked plasma (After extraction)	22.05	286.11	1951.99
% Mean recovery	95.63	97.24	98.17

analysis. From a range of different buffers investigated, ammonium dihydrogen phosphate provided the required symmetry of peaks. Moreover, a mobile phase having the composition of ammonium dihydrogen phosphate buffer and methanol (60 : 40) with pH 3.5 produced inadequate peak symmetry. Decreasing pH to 2.5 and increasing the ratio of buffer to 98% produced peak with good resolution and symmetry Thus, a mobile phase consisting of ammonium dihydrogen phosphate buffer and methanol (98 : 2) with pH 2.5 adjusted with orthophosphoric acid was considered optimum with respect to peak shape, retention time and sensitivity. Investigation of flow rates was done between 0.5 and 1.5 mL/min. It has been found that an optimal and reasonable separation time has been obtained by keeping a flow rate of 1 mL/min. Moreover, the retention time of ACV in the present method was detected to be 6.3 min whereas total analysis time was kept 10 min. The UV-visible scan was conducted at 500-200 nm and peak response was recorded. The maximum absorption of ACV was noted at 256 nm and this wavelength was chosen for analysis. The temperature during analysis was controlled at 25°C and pressure was maintained at 108-116 bars. Thus on the basis of above observations, mobile phase composition and ratio of organic and aqueous phase, flow rate, pH and wavelength were chosen to give optimum retention times with a good resolution.

Optimization of extraction of ACV from plasma samples was done considering the number of factors like simplicity of the procedure, good recovery, reproducibility while keeping the processing steps to a minimum level (25-26). The recoveries were observed to be minimum representing high concentration or processing losses when organic solvents such as dichloromethane, chloroform and *n*-hexane were tested (27). However, when extraction was conducted using perchloric acid, it has been observed that perchloric acid has resulted in good recovery. Therefore, perchloric acid has been selected as a participant for extraction. Table 1 summarizes the overall chromatographic conditions of the developed method.

Method validation

Selectivity

Chromatograms of ACV in mobile phase, blank plasma and spiked plasma are shown in Figure 1A, 1B and 1C respectively. It has been observed in each case that no interference of endogenous peaks from plasma was present with the elution of the ACV. ACV peaks in all cases were well resolved with good symmetry and reasonable retention time of 6.3 min. This confirms excellent separation under selected chromatographic conditions and the selectivity of the method (28-29).

Standard curve and linearity

Construction of the standard curve was done between the ACV peak areas versus the corresponding ACV concentration in plasma. Three different

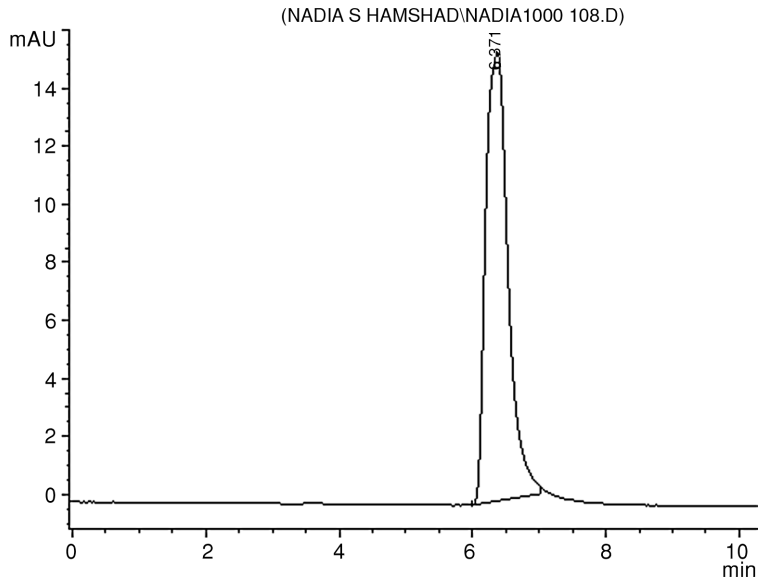

Figure 1A. Chromatogram of acyclovir from a dilution of stock solution in mobile phase

standard curves have been constructed on three different days and have been observed to be linear over the range of 25-2000 ng/mL by linear regression analysis. Table 2 summarizes regression correlation equations for calibration curves and their correlation coefficients. The correlation coefficients of all standard curves were found to be equal to or greater than 0.998.

Precision and accuracy

Both inter-day and intra-day precision and accuracy of the standard curve have been investigat-

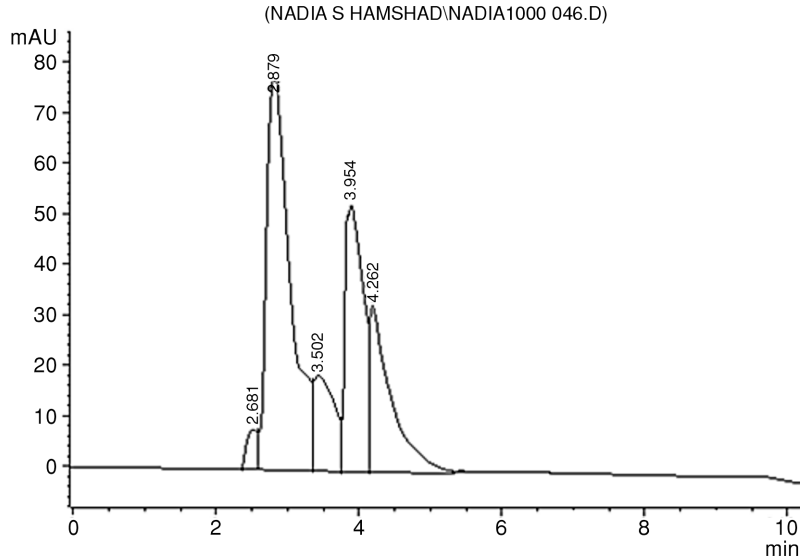

Figure 1B. Chromatogram of blank rabbit plasma

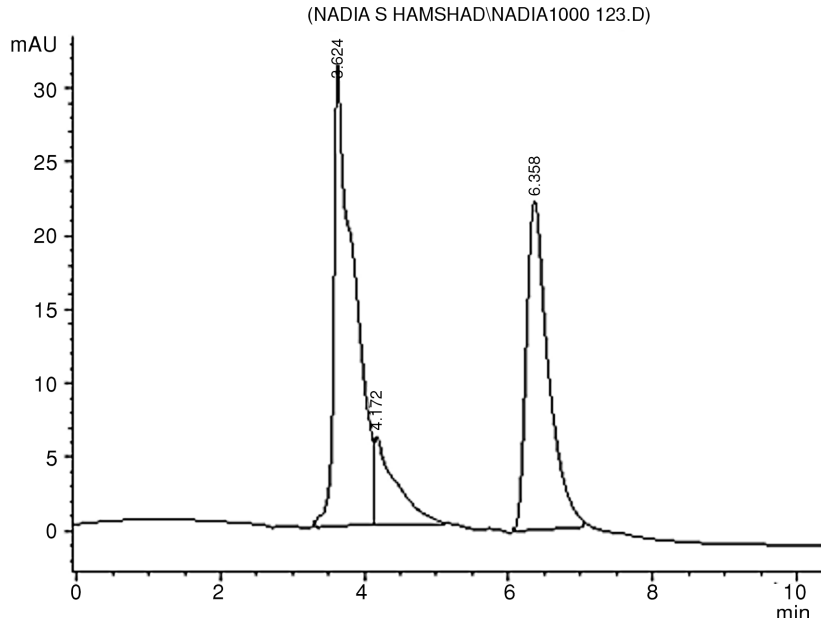

Figure 1C. Chromatogram of spiked rabbit plasma

Table 5. Stability of ACV in stock solution and plasma under various conditions (n = 5).

Storage conditions	Acyclovir calculated concentration (ng/mL)	
	Mean stability ± SD	% Change
Short term stock solution stability (6 h) at room temperature	99.878 ± 76	-0.121
Long term stock solution stability 2-8°C for 6 days	99.638 ± 258	-0.369
Bench top stability		
HQC (2000 ng/mL)	1849.71 ± 31.61	-9.49
MQC (300 ng/mL)	280.56 ± 7.40	-6.48
LQC (25 ng/mL)	20.83 ± 0.98	-7.51
Freeze & thaw stability		
HQC (2000 ng/mL)	1916.46 ± 45.64	-4.17
MQC (300 ng/mL)	276.50 ± 17.57	-7.83
LQC (25 ng/mL)	21.75 ± 1.76	-8.97
Long term stability (-20°C) for 4 weeks		
HQC (2000 ng/mL)	1809.22 ± 52.11	-8.53
MQC (300 ng/mL)	268.70 ± 13.10	-7.7
LQC (25 ng/mL)	20.78 ± 1.30	-9.65

ed with high, medium and low QC controls. As can be seen from Table 3 that precision and accuracy values have not deviated by ±15.0%, representing ruggedness of the method (30) and sufficient accuracy of assay for its application in biostudies (31).

Recovery

Optimization of an extraction method for ACV from plasma samples was done by keeping processing steps minimum, reproducible and as simple as possible. Perchloric acid was used as a precipitant for conducting extraction. The % mean recovery of ACV measured with low, medium and high concentration levels was estimated to be 95.63, 97.24 and 98.17, respectively as shown in Table 4. Thus it has been indicated that protein precipitation with 5% perchloric acid can be considered a valid and reliable method to eliminate plasma protein, giving high % mean recovery (32).

Sensitivity

The lowest limit of detection, LLOD was found by carrying out dilution of solutions having known concentration till the response was estimated three times the noise. The lowest limit of detection of ACV was indicated to be 15 ng/mL. Hence, we can say it is the amount of ACV in standard solution and spiked plasma samples which we can detect only but are unable to quantify or estimate. Moreover, LLOQ defined as lower limit of quantification was found to be 25 ng/mL. The 25 ng/mL is the lowest concentration of ACV that we can estimate or quantify. Hence for the above reasons, this is considered as the lowest concentration to be used for the construction of calibration curve for a standard solution and plasma samples and in determination of precision and accuracy. However, in some other studies conducted previously (10), the lower limit of detection for ACV and lower limit of quantification was found to be much lower, representing more sensitivity of their developed method in comparison to the value of LLOD and LLOQ determined in our method. The variation in detection might be due to the use of high-performance capillary electrophoresis for drug detection, while in our method the ACV has been detected through UV detector.

Stability

The results of stability experiments conducted for the stock solution and plasma samples have been indicated in Table 5. It has been clearly indicated that stock solution of ACV was found to be stable when stability experiment was conducted for short term stability of minimum 6 h at room temperature and long term stability of 6 days between 2-8°C respectively. Results of benchtop stability at room temperature, long term stability of samples stored at -20°C up to four weeks and freeze and thaw stabili-

ty for low, medium and high-quality control plasma samples showed them to be stable at their respective conditions. Furthermore, deviation of mean calculated concentrations of samples was found to be within 15% when compared with freshly made quality control samples.

CONCLUSION

HPLC method for the quantitation of acyclovir in rabbit plasma was developed and validated as per FDA guidelines. The developed HPLC method is simple, eliminated endogenous interferences and shown to be robust for bioassay. By optimizing the chromatographic conditions, a LOQ of 25 ng/mL is achieved, representing high sensitivity of the validation method. Moreover, the method exhibited linearity within a concentration range of 25-2000 ng/mL with precision, accuracy, sensitivity, and selectivity. Hence method described could be used for the determination of ACV in bioavailability and bioequivalence studies.

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Conflict of interest statement

The author reports no conflict of interest.

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