



ORIGINAL ARTICLE

A newfangled study using risk silhouette and uncertainty approximation for quantification of acyclovir in diverse formulation



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Abstract Risk assessment and uncertainty approximation are two major and important parameters that need to be adopted for the development of pharmaceutical process to ensure reliable results. Additionally, there is a need to switch from the traditional method validation checklist to provide a high level of assurance of method reliability to measure quality attribute of a drug product. In the present work, evaluation of risk profile, combined standard uncertainty and expanded uncertainty in the analysis of acyclovir were studied. Uncertainty was calculated using cause-effect approach, and to make it more accurately applicable a method was validated in our laboratory as per the ICH guidelines. While assessing the results of validation, the calibration model was justified by the lack of fit and Levene's test. Risk profile represents the future applications of this method. In uncertainty the major contribution is due to sample concentration and mass. This work demonstrates the application of theoretical concepts of calibration model tests, relative bias, risk profile and uncertainty in routine methods used for analysis in pharmaceutical field.

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1. Introduction

Measurements are made and used to make decisions. Whenever we use numbers to make decisions, we run the risk of making a mistake because all numbers are more or less unreliable. If we measure something, we can be certain of only one thing: the

measurement is dicey. Since every measurement is doubted, we should know how and why it is so. With the contemporary development of analysis field errors, doubted results are also increasing. Therefore, to avoid these doubts recently a newer approach has been introduced known as measurement of uncertainties in analysis of a sample. As the result of measurement is only an estimation of the measurand value (μ), it is accepted only when accompanied by a quantitative statement of its uncertainty (U) and is expressed as $(\mu+U)$. Managing uncertainty is a basic effort in analytical method development and validation. The

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quality of method is expressed as its uncertainty and evaluation of uncertainty nowadays has become an important parameter for a validated method to get accreditation [1,2].

In 2012, Eurachem introduced the third edition of guidelines for quantifying uncertainty in analytical measurement based upon developments in uncertainty estimations [3]. The most important characteristic of a properly calculated uncertainty budget, compared to other measures of method performance, is that it encompasses on both random and systematic effects to give a single value. For the uncertainty estimation, the steps involved start with measurand specification and end with expanded uncertainty (EU) calculation.

Nowadays, heaps of analytical methods have been introduced and published worldwide for the estimation of drug contents in pharmaceutical formulations. But as literature suggests and to our best knowledge, validation by total error approach and quantification of sources of uncertainties in these methods have been missing. However, a few methods are there, but they are lacking in explanation of simple ways for quantifying uncertainty components, combined standard uncertainty (CSU) and EU. Thus to enlighten the total error approach in pharmaceutical field, in the current study a simple methodology for quantification of uncertainty components and CSU is presented by assessing these computations for spectrophotometric measurement of acyclovir in its different formulations. To have a detailed and accurate study of uncertainty components, a new UV method for acyclovir and its different dosage forms has been developed and validated as per the ICH guidelines [4,5]. β -expectation tolerance interval, relative bias, accuracy profiles, risk profile and calibration model were studied. This approach also includes detailed analysis of the factors influencing analytical results using cause-effect diagram, and risk profile of future assessment.

Acyclovir (9-[(2-hydroxyethoxy)-methyl]-guanine) is the most widely used antiviral agent in our community. It is an acyclic guanosine derivative with clinical activity against HSV-1, HSV-2 and against varicella-zoster virus. It has also been used in the treatment of primary and recurrent genital herpes, herpes simplex encephalitis and neonatal HSV infection [6–8]. It has been shown that acyclovir has high solubility and low intestinal permeability and considered as a typical class III drug according to The Biopharmaceutics Classification System (BCS) from the Food and Drug Administration (FDA) of the United States [9,10]. The therapeutic importance of acyclovir has promoted the development of many analytical methods for its quantitative determination. These methods include high-performance liquid chromatography (HPLC) [11–21], micellar liquid chromatography [22], gas chromatography [23], capillary electrophoresis [24], and radioimmunoassay [25]. Spectrophotometric analyses are considered as more convenient alternative techniques because of their inherent simplicity and high sensitivity. Since, acyclovir contains a weakly absorbing chromophore, few spectrophotometric methods [26–29] have been reported for their determination, but all these methods are laborious, time consuming or/and require derivatization of the drug.

Uncertainty estimation and β -expectation tolerance interval, relative bias, accuracy profiles, risk profile and calibration model, which were not studied either, are critical parameters in today's method validation procedures. Some articles propose the conventional estimation of analytical measurements and uncertainty as well [30–33]. However, most of these methods are applied to food samples and a very few methods have been found for pharmaceutical formulations [34]. Therefore, we developed a new simple

spectrophotometric method that overcomes these drawbacks by applying a wide uncertainty estimation and total error estimation approach.

2. Experimental

2.1. Instrumentation

Spectrophotometric measurements were made on a Shimadzu 1700 double beam UV–vis spectrophotometer with a fix slit width of 1 nm coupled with Shimadzu UV PC software (UV probe) version 2.31. Weighing balance of Shimadzu AX120, bath sonicator (Electroquip) and borosil glass apparatus were used for experimental purpose.

2.2. Materials and reagents

Pure acyclovir was procured as gift sample from Nestor Pharmaceutical Pvt. Ltd., India. Various commercial formulations of acyclovir were purchased from local drug store. All the reagents used in this study were of analytical grade. Double distilled water was used for the preparation of the solutions, and for robustness studies HPLC grade and single distilled water were used.

2.3. Preparation of standard solutions

An accurately weighed amount of acyclovir was transferred into a 10 mL calibrated flask and dissolved in approximately 4 mL of 0.1 M HCl. The resulting solutions were completed to the mark with 0.1 M HCl obtaining stock standard solution containing 1000 $\mu\text{g/mL}$. Different volumes of this stock solution were then further diluted with 0.1 M HCl to obtain the working standard solutions.

2.4. Sample preparation for different formulations

2.4.1. Optimization of sonication time

Sonication time used in the preparation of samples of different formulations was optimized for all the different matrices by trial methods.

2.4.2. Tablets

Twenty tablets were finely powdered and an accurately weighed quantity of the powdered tablets content equivalent to 10 mg of the active ingredient was transferred into a 10 mL calibrated flask and dissolved in about 6 mL of 0.1 M HCl. The contents of the flask were swirled, sonicated up to 9 min and then volume of the flask was made up with 0.1 M HCl. The contents were mixed well, filtered and the first portion of the filtrate was rejected. The prepared solution was diluted quantitatively with the 0.1 M HCl to obtain a suitable concentration for analysis.

2.4.3. Cream samples

An accurately weighed amount of the cream equivalent to 10 mg of acyclovir was shaken with 5 mL of 0.1 M HCl and sonicated for 15 min and then the volume was made up to the mark with 0.1 M HCl. The resulting solution was filtered and the first portion of the filtrate was discarded. The working solutions were prepared by further diluting with 0.1 M HCl for analysis.

2.4.4. Eye ointment sample

Eye ointment equivalent to 10 mg of acyclovir was dispersed in few mL of 0.1 M HCl, sonicated for 18 min and the volume was made up with 0.1 M HCl. The resulting solution was filtered, and the first portion of the filtrate was rejected. The working solution was prepared by further diluting with 0.1 M HCl for analysis.

2.4.5. Injection

To determine the drug in injectable dosage form, the contents per injection were diluted with 0.1 M HCl and then sonicated for 9 min and the volume was made up with 0.1 M HCl. The resulting solution was filtered, and the first portion of the filtrate was rejected. The working solution was prepared by further diluting with 0.1 M HCl for analysis.

2.5. Procedure for spectrophotometric determination

All reagents were tested for stability in solution and during the actual analysis. The behavior of analyte remained unchanged for about 24 h from their preparation at room temperature. The drug was found to be stable during each kind of experimental measurements. Each measurement was done at room temperature. The absorption spectra of the standard solutions were recorded between 200–400 nm against a reagent blank (the same for samples without the analyte to be determined) using a 1.0 cm quartz cell. The zero-order spectrum of pure drug was obtained and absorption maxima was found to be at 257 nm.

2.6. Validation parameters studied using total error approach

The present method was validated according to the ICH [4,5] and the ISO-17025 applying accuracy profiles, which are based upon the “total error” approach [1]. This approach estimates the “total error” by combining the systemic error (trueness) and the random error (intermediate precision) to know the difference between the observed result and the true value. In other words, the highest error of an analytical method can be estimated. In the proposed method different parameters such as sensitivity, robustness and assay determination were also studied.

2.7. Response function (calibration curve)

In the proposed method four sets of calibration curve were plotted between absorbance and different concentrations of acyclovir which follows *Lambert–Beer’s* law and on these four different series regression analysis was performed and the series with best coefficient of determination was selected and the selected linearity has been diagnosed by the Lack of Fit, Levene’s test and standard residual plot.

2.8. Trueness

According to the ISO, trueness of an analytical procedure expresses the closeness of agreement between the average value obtained from repeated measurements and a conventional true value [35]. In this method trueness of calibration curve is calculated to justify the calibration line by back calculating concentrations and results are expressed in terms of absolute and relative bias, and also a linear relationship between introduced and back calculated concentrations has been plotted to demonstrate method linearity.

2.9. Precision

The precision of an analytical method expresses the closeness of agreement between the values obtained from repeated measurements. In this method precision at two levels was studied: the first one was repeatability under the same operating conditions over a short time interval and the second was the intermediate precision assessed on different days. The precision results are expressed using relative standard deviations (RSD). Relative precision and absolute precision at these two levels were calculated and also 95% upper confidence limit for both levels was calculated.

2.10. Accuracy

Accuracy is the most critical parameter in method validation, so it requires an extra care during the study. Therefore, the results of accuracy studies were represented in the β -expectation tolerance limits. In addition to this, risk profile was also studied to know the future application of the method in different matrices. Accuracy profiles for the four different matrices were plotted with β -expectation tolerance limits. Linearity profile was also studied to demonstrate the relationship between nominal and observed concentrations in different matrices and furthermore, residual plot was generated to know the outliers in the determination of acyclovir in sample matrix.

2.11. Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) are two important parameters which show the application of methods in quantification and detection of different samples. These were calculated according to the procedure mentioned in the ICH guidelines [4,5].

2.12. Robustness

In daily routine analysis some human or system errors are always there in sample preparation and system measurement properties. A method should be steady to avoid these errors or small variations and should not deviate from its capability of producing reliable results. So, to confirm this robustness studies were performed. Robustness was examined by evaluating the influence of small variation of method variables including solvent grades and detection wavelengths. In these experiments, one parameter was changed whereas the others were kept unchanged and recovery percentage was calculated each time and for all the formulations, recovery experiments for robustness studies were also planned and studied. The robustness studies were conducted with three different wavelengths and three different grades of solvent (HPLC, SD, DD). The study was conducted by keeping one standard parameter constant and varying the second factor respectively.

2.13. Application of proposed method to analysis of dosage forms

After the confirmation of method capacity to analyze acyclovir, it was subjected to the analysis of different formulations for their contents of acyclovir. The results for different matrices were calculated in terms of percentage purity. These results confirmed the capacity and reliability of the developed method.

Table 1 Results of LOF and Levene's test for linear regression model.

Test	Error	SS	df	MS	F_{calc}	$F_{crit, 95\%}$	p -Value
Lack of Fit	LOF error	0.0001047	16	0.00000873	1.775	1.918	0.1046
	Pure error	0.0001475	38	0.00000492			
Levene's	Model	0.0000142	5	0.00000285	1.886	2.380	0.1173
	Error	0.0000634	56	0.00000151			

equation Eq. (5).

$$Y = mx + c \quad (5)$$

where Y , absorbance of sample; c , calibration curve intercept; m , calibration curve slope; x , concentration of acyclovir.

2.15.4. Sample mass (m_{sample})

The sample mass was obtained by calculating difference between weighing glass with and without the acyclovir sample.

2.15.5. Precision of method (P)

The precision branch collects terms which contribute to random variability of the entire method. Estimate of precision is available from replicate analysis of samples. When the precision studies were carried out and data were obtained, the repeatability and variability associated with that measurement were included in the overall precision uncertainty estimation.

3. Results and discussion

The absorption spectrum of acyclovir was recorded and it shows the maximum absorption intensity at 257 nm. Thus all the studies were carried out at the same wavelength (nm).

3.1. Validation parameters

3.1.1. Response function (calibration curve)

In the proposed method calibration curves were prepared using linear regression model. Four different sets were prepared for response function studies within the range of acyclovir from 2 to 12 $\mu\text{g/mL}$. As four sets were prepared, all of them were found to follow the linear regression model and their regression analysis parameters results were studied. As from regression analysis studies, series 4 shows the best coefficient of determination r^2 as 0.9999 with regression equation ($Y=0.08083X-0.001444$), which was selected for further studies and computation. Furthermore, the linear regression model was also confirmed for its suitability for method by diagnosis using Lack of Fit (LOF) test and Levene's test. As p -values were found to be higher than 0.05, represented in Table 1 to demonstrate that no outliers were found in calibration curve, standard residual plot was also plotted as shown in Fig. 2. Now to confirm the chosen regression equation, back calculation was done and linear plot based upon absolute β -expectation limit was generated between nominal and back calculated concentration which shows r^2 value is 0.9998 and it becomes clear that the calibration lines adequately describe the observed relationship.

3.1.2. Trueness

To justify the trueness of the method percentage relative bias was calculated and is illustrated in Table 2 from where it can be concluded that the trueness for all different concentrations is

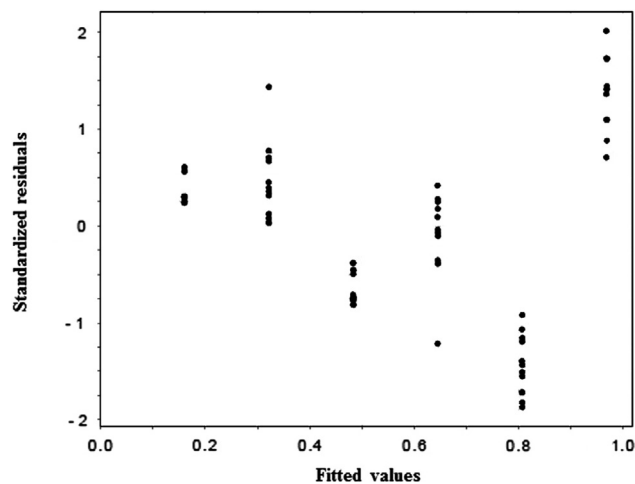


Fig. 2 Standard residual plot of four different series representing absence of outliers in all different concentration levels.

Table 2 Results of trueness in terms of relative bias (%).

Nominal concentration ($\mu\text{g/mL}$)	Back calculated concentration ($\mu\text{g/mL}$)	Absolute bias ($\mu\text{g/mL}$)	Relative bias (%)
2.000	2.038	0.03754	1.877
4.000	3.952	-0.04821	-1.205
6.000	5.974	-0.02588	-0.4313
8.000	7.949	-0.05130	-0.6413
10.00	10.04	0.03764	0.3764
12.00	12.00	0.000905	0.00754

acceptable, since the percentage relative bias is limited between -0.641% and 1.87% .

3.1.3. Precision

The results of precision were found to be $<2\%$ in terms of RSD for both repeatability and intermediate levels. These results of intermediate and repeatability precision suggest that the developed analytical method was precise and reproducible. The results of relative and absolute intermediate precision and repeatability are shown in Table 3 which justifies the reproducibility of method and further 95% confidence upper limit has been demonstrated in Table 3 for both intermediate precision and repeatability.

3.1.4. Accuracy

The accuracy of the method was carried out by standard addition method. Accuracy takes into account the total error of the test results and is represented by the β -expectation tolerance limits. The method accuracy was performed using different matrices

Table 3 Results of relative and absolute intermediate precision and repeatability in terms of %RSD.

Nominal concentration ($\mu\text{g/mL}$)	Relative intermediate precision and repeatability				Absolute intermediate precision and repeatability			
	Rep ^a (%RSD)	IP ^b (%RSD)	95% Upper confidence limit		Rep ^a (SD) ($\mu\text{g/mL}$)	Between-series (SD) ($\mu\text{g/mL}$)	Ration of variance components (between/within)	IP ^b (SD) ($\mu\text{g/mL}$)
			Rep ^a (SD) ($\mu\text{g/mL}$)	IP ^b (SD) ($\mu\text{g/mL}$)				
2.000	0.4252	0.5263	0.0161	0.0663	0.1183	0.0677	0.0136	0.0405
4.000	0.4857	0.5657	0.0353	0.0353	0.0473	0.0184	0.1521	0.0507
6.000	0.4184	0.5586	0.0937	0.0513	0.0395	0.0226	0.3277	0.0455
8.000	0.2134	0.2877	0.0662	0.0770	0.0227	0.0039	0.0304	0.0230
10.00	0.2544	0.3493	0.0822	0.0566	0.0514	0.0193	0.1402	0.0549
12.00	0.2152	0.3619	0.0750	0.0697	0.0630	0.0239	0.1441	0.0674

^aRep – Repeatability.^bIP – Intermediate precision.**Table 4** Result of method accuracy in terms of relative beta-expectation tolerance limit and risk assessment obtained by selected regression model in different matrices.

Matrix	Concentration level (%)	Concentration ($\mu\text{g/mL}$)	Beta- expectation tolerance limits ($\mu\text{g/mL}$)	Relative beta- expectation tolerance limits (%)	Risk ^a (%)
Tablet	80.0	8.000	[7.91, 8.10]	[−1.17, 1.30]	0.000339
	100.0	10.00	[9.89, 10.09]	[−1.10, 0.90]	0.000048
	120.0	12.00	[11.95, 12.06]	[−0.44, 0.52]	0.00000003
Cream	80.0	8.000	[7.92, 8.13]	[−1.02, 1.65]	0.000832
	100.0	10.00	[9.79, 10.10]	[−2.06, 1.05]	0.003895
	120.0	12.00	[11.85, 12.20]	[−1.24, 1.66]	0.001583
Ointment	80.0	8.000	[7.86, 8.09]	[−1.78, 1.08]	0.001602
	100.0	10.00	[9.97, 10.15]	[−0.34, 1.45]	0.000029
	120.0	12.00	[11.75, 12.19]	[−2.05, 1.59]	0.01124
Injection	80.0	8.000	[7.92, 8.13]	[−0.9815, 1.623]	0.000667
	100.0	10.00	[9.80, 10.09]	[−1.970, 0.9440]	0.002294
	120.0	12.00	[11.85, 12.21]	[−1.281, 1.708]	0.002071

^aRisk of having measurements falling outside of the acceptance limits ($\pm 5\%$).

and thus accuracy obtained by considering linear regression model has been summarized in Table 4. It was also found that the β -expectation tolerance limits do not exceed the acceptance limits which means that β -percent (95%) of the future measurement of unknown samples will be included within the tolerance limits as shown by accuracy profile illustrated in Fig. 3. Accuracy profile of the method was also justified by risk profile by choosing maximum risk level at 5.0% and it was concluded that the risk of outliers are within limits and future analysis of unknown sample will fall within the range. To evaluate the errors in intra accuracy studies a linear plot was also generated which shows the linearity between nominal and observed concentrations with r^2 as 0.9989 and also confirms the outliers in different spiking concentrations. A standard residual plot was plotted, which shows that there are no outliers falling in the intra accuracy studies as shown in Fig. 4.

3.1.5. Limit of detection and quantification

Results of LOD and LOQ show that this method is sensitive enough to analyze the marketed formulations. Values of LOD and LOQ were found to be 0.255 and 0.772 $\mu\text{g/mL}$, respectively.

3.1.6. Robustness studies

Robustness of the developed method was determined in the form of percentage RSD by small but deliberate changes in the solvent grade and detection wavelength in all the sample matrices. The results of robustness studies are represented in Table 5 showing the effect of variation on amount found in sample matrix and from these results it is concluded that the method has enough capacity to bear up to some extent human or system errors.

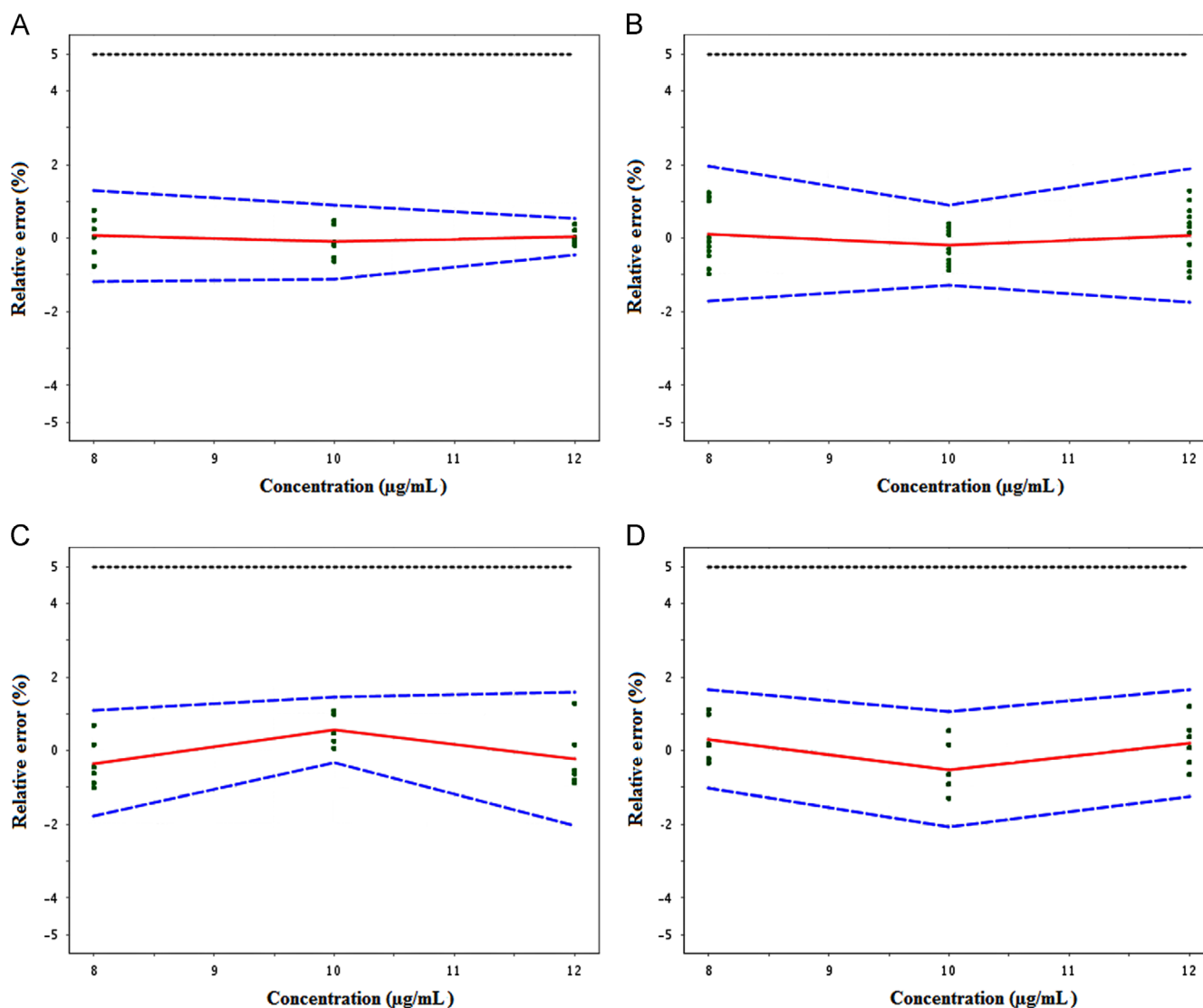


Fig. 3 Accuracy profile of acyclovir determination in (A) tablets, (B) skin cream, (C) eye ointment, (D) injection obtained after application of linear regression using calibration standards prepared with the matrix. The plain line is the relative bias, the dashed lines are the 95% β -expectation tolerance limits and the dotted curves represent the acceptance limits ($\pm 5\%$). The dots represent the relative back-calculated concentrations of the validation standards.

3.2. Application of the proposed method to analysis of dosage forms

3.2.1. Sonication time optimization

As the matrices are different, the extraction time of acyclovir from them will be different. To achieve the maximum extraction of acyclovir, optimization of sonication time was carried out by analyzing samples after different sonication times. The most suitable sonication time for tablet, skin cream, eye ointment and injection was found to be 9, 15, 20, and 9 min, respectively. After sonication for these particular time period, percentage purity for all formulations was found to be in the range of 99–101%.

3.2.2. Analysis of dosage form

It is evident from the aforementioned results that the proposed method gave satisfactory results with the drug. Thus dosage forms were subjected to analysis for their contents of active drug material by the proposed method. The percentage purity

for skin cream, eye ointment, tablet and injection was found to be 99.75, 101.36, 100.9 and 99.21, respectively. It is evident from the above mentioned values that the proposed method is applicable to the analysis of drug in its bulk and dosage forms with comparable analytical performance. The critical recommendations of this method might be based on their relative sensitivities (that determines the amount of specimen available for analysis) and experimental conditions (sonication time, diluting solvent, etc.).

3.3. Measurement of uncertainty

After the uncertainty sources were identified according to the cause-effect diagram, they were evaluated, their magnitude was determined, and in order to assure the traceability for uncertainty results all the calculations were done in the International System of Units as concentration in M and weight in kg.

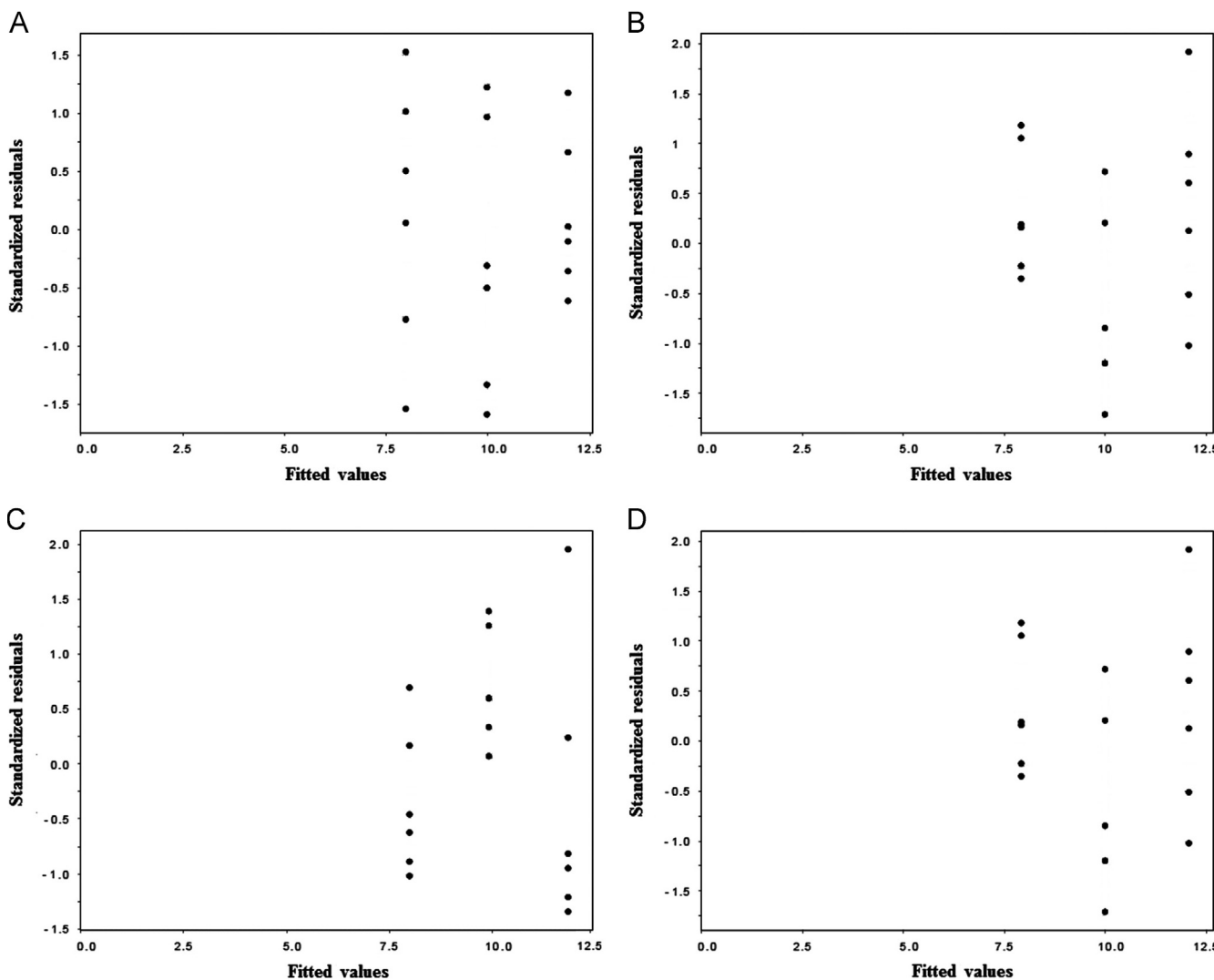


Fig. 4 Standard residual plot confirming absence of outliers in determination of accuracy of (A) tablets, (B) skin cream, (C) eye ointment and (D) injection.

3.3.1. Uncertainty due to concentration (C_{10})

After the scanning of standard solution, values in terms of absorbance were obtained and calibration curve was plotted as described by Eq. (5). Regression equation of calibration curve was identified as slope 10,746 and intercept 0.079. For the determination of calibration curve, six solutions were measured three times (total number of measurements, $n=18$). The sample solution was measured 10 times, thus obtaining analyte concentration in tablet, cream, ointment and injection. Results are represented in Table 6.

Thus:

$$S_{xx} = 1.142 \times 10^{-9} \quad (6)$$

$$S_r = 3.037 \times 10^{-2} \quad (7)$$

Then using S_r and S_{xx} , standard relative uncertainty due to concentration was calculated and results are expressed in Table 7. A very small difference was observed in the standard relative uncertainty of different formulations.

3.3.2. Uncertainty of the liberation of 10 mL volume of 10 mL volumetric flask

The effect on volume of 10 mL volumetric flask is mainly influenced by the three parameters, i.e. calibration of the volumetric flask (at the time of manufacturing), repeatability and temperature.

3.3.2.1. Calibration of volumetric flask. Deviation of value from nominal volume for 10 mL volumetric flask was ± 0.007 mL (at 27 °C) as given by the manufacturer and by assuming that standard deviation is not claimed by the manufacturer with confidence interval limit, standard value of uncertainty can be calculated with triangular distribution. Thus, uncertainty associated with liberation of 10 mL volume of 10 mL volumetric flask due to calibration $u(V_{10cal})$ is shown in Eq. (8).

$$u(V_{10cal}) = \frac{0.007}{\sqrt{6}} = 2.86 \times 10^{-3} \text{ mL} \quad (8)$$

3.3.2.2. Repeatability $u(V_{10rep})$. After filling and weighing 10 mL volumetric flask, standard uncertainty of volumetric flask was established at 0.0014 mL.

Table 5 Result of robustness studies in different variations in terms of mean concentration found and %RSD ($n=6$).

Parameters studied		Nominal concentration ($\mu\text{g/mL}$)		Mean concentration found ($\mu\text{g/mL}$) \pm %RSD			
				Tab.	Crm.	Ont.	Inj.
Solvent grade	HPLC	10		9.98 \pm 0.79	10.01 \pm 0.32	9.99 \pm 0.35	9.98 \pm 0.35
	SD ^a	10		9.96 \pm 0.44	9.94 \pm 0.56	9.98 \pm 0.71	10.01 \pm 0.65
	DD ^b	10		9.93 \pm 0.64	9.93 \pm 0.33	10.02 \pm 0.51	9.99 \pm 0.53
λ max (nm)	256	10		9.96 \pm 0.60	9.91 \pm 0.41	9.97 \pm 0.80	9.93 \pm 0.59
	257	10		9.95 \pm 0.91	9.91 \pm 0.91	9.98 \pm 0.42	9.99 \pm 0.63
	258	10		9.95 \pm 0.90	9.96 \pm 0.90	9.97 \pm 0.86	9.96 \pm 0.42

Tab – Tablet, Crm – Skin cream, Ont – Eye ointment, Inj – injection.

^aSD – Single distilled,

^bDD – Double distilled.

Table 6 Results of concentration determination of acyclovir in tablet, skin cream, eye ointment and injection.

Sample no	Acyclovir found (g)				Concentration ($\text{M} \times 10^{-5}$)			
	Tablet	Skin cream	Eye ointment	Injection	Tablet	Skin cream	Eye ointment	Injection
1	0.01007	0.00982	0.00973	0.00996	4.08	3.97	3.94	4.03
2	0.01010	0.00977	0.00998	0.00998	4.08	3.95	4.04	4.04
3	0.01012	0.00987	0.00977	0.00994	4.09	3.99	3.95	4.02
4	0.01000	0.01005	0.01010	0.00989	4.05	4.07	4.08	4.00
5	0.01003	0.00996	0.00973	0.00987	4.06	4.03	3.94	3.99
6	0.01005	0.01007	0.01014	0.01005	4.07	4.08	4.10	4.07
7	0.00994	0.01012	0.01000	0.00994	4.02	4.09	4.05	4.02
8	0.00998	0.01000	0.00996	0.00998	4.04	4.05	4.03	4.04
9	0.01007	0.00973	0.00998	0.01007	4.08	3.94	4.04	4.08
10	0.00996	0.01007	0.00973	0.00996	4.03	4.08	3.94	4.03
Mean	0.01003	0.00995	0.00991	0.00996	4.06	4.02	4.01	4.03

3.3.2.3. *Temperature.* The manufacturer has calibrated volumetric flask at the time of manufacturing at a temperature of 27 °C, while temperature in the laboratory varied within a range of $\Delta t = \pm 4$ °C. This difference was overcome by calculating uncertainty value with estimation of temperature range and volume dilatation coefficient. Volume expansion of liquid was taken into consideration, as it is quite higher than expansion of volumetric flask. The volume expansion coefficient, γ , of water is $2.1 \times 10^{-4}/\text{°C}$. Thus uncertainty for 10 mL volumetric flask ΔV_{10} was calculated by Eq. (9).

$$\Delta V_{10} = V_{10} \times \gamma \times \Delta t \quad (9)$$

where ΔV_{10} , uncertainty of the 10 mL volumetric flask; V_{10} , volume of the 10 mL volumetric flask; γ , volume dilatation coefficient; Δt , temperature variation in the laboratory.

Thus, we obtain that uncertainty for volumetric flask of 10 mL is 0.0084 mL, also assuming temperature variation is rectangular distribution, standard uncertainty for 10 mL volumetric flask due to the temperature effect will be $u(V_{10-temp})$ as shown in Eq. (10).

$$u(V_{10-temp}) = \frac{4 \times 2.1 \times 10^{-4} \times 10}{\sqrt{3}} = 0.0048 \text{ mL} \quad (10)$$

Thus, standard uncertainty due to liberation of 10 mL volume of 10 mL volumetric flask was calculated according to Eq. (11) and

was found to be 0.0058 mL. Standard relative uncertainty was calculated and shown in Eq. (12).

$$u(V_{10}) = \sqrt{(u(V_{10-cal}))^2 + (u(V_{10-rep}))^2 + (u(V_{10-temp}))^2} \quad (11)$$

$$u(V_{10}) = 0.0058 \text{ mL}$$

The standard relative uncertainty will be:

$$\frac{u(V_{10})}{V_{10}} = 5.76 \times 10^{-4} \quad (12)$$

3.3.3. Uncertainty associated with the sample mass (m_{sample})

Estimation of sample mass has three types of uncertainty sources such as sensitivity, linearity, and repeatability. Mass of the sample was expressed in kg for convenient traceability of results.

3.3.3.1. *Sensitivity.* The range of difference in weighed mass was small and the same weighing balance was used each time. Thus, uncertainty due to sensitivity of balance can be neglected.

3.3.3.2. *Linearity.* As the manufacturer data indicated a linearity value of 0.0001 g, to determine overall uncertainty value, standard uncertainty due to linearity was considered. A rectangular

Table 7 Summary of contribution to the measurement uncertainty.

Formulation	Parameter	Volume, V_{10} (mL)	Sample concentration C_{10} (M)	Mass sample, m_{sample} (kg)	Recovery method	Repeatability
Tablet	Value	10	4.058×10^{-8}	1.003×10^{-5}	99.63×10^{-2}	–
	Standard uncertainty, $u(x)$	5.76×10^{-3}	1.15×10^{-9}	2.91×10^{-7}	1.98×10^{-2}	3.30×10^{-3}
	RSU, $u(x)/x$	5.76×10^{-4}	3.73×10^{-2}	2.9×10^{-2}	1.99×10^{-2}	3.30×10^{-3}
Eye ointment	Value	10	4.01×10^{-8}	9.91×10^{-6}	99.75×10^{-2}	–
	Standard uncertainty, $u(x)$	5.76×10^{-3}	1.49×10^{-9}	2.91×10^{-7}	1.98×10^{-2}	3.3×10^{-3}
	RSU, $u(x)/x$	5.76×10^{-4}	3.71×10^{-2}	2.94×10^{-2}	1.99×10^{-2}	3.3×10^{-3}
Skin cream	Value	10	4.02×10^{-8}	9.95×10^{-6}	10.10×10^{-1}	–
	Standard uncertainty, $u(x)$	5.76×10^{-3}	1.49×10^{-9}	2.91×10^{-7}	2.03×10^{-2}	3.30×10^{-3}
	RSU, $u(x)/x$	5.76×10^{-4}	3.71×10^{-2}	2.93×10^{-2}	2.01×10^{-2}	3.30×10^{-3}
Injection	Value	10	4.03×10^{-8}	9.96×10^{-6}	99.50×10^{-2}	–
	Standard uncertainty, $u(x)$	5.76×10^{-3}	1.50×10^{-9}	2.91×10^{-7}	2.0×10^{-2}	3.30×10^{-3}
	RSU, $u(x)/x$	5.76×10^{-4}	3.73×10^{-2}	2.93×10^{-2}	2.01×10^{-2}	3.30×10^{-3}

distribution was assumed to convert contribution of linearity. It was calculated and is expressed in Eq. (13).

$$u = \frac{0.0001 \times 10^{-3}}{\sqrt{3}} = 5.77 \times 10^{-8} \text{ kg} \quad (13)$$

3.3.3.3. *Repeatability.* Uncertainty associated with repeatability was found to be 0.00028×10^{-3} kg.

3.3.4. *Computation of relative uncertainty due to sample mass* Uncertainty due to sample mass $u(m_{sample})$ was calculated as shown in Eq. (14).

$$u(m_{sample}) = \sqrt{2 \times (5.77 \times 10^{-8})^2 + (0.00028 \times 10^{-3})^2} = 2.91 \times 10^{-7} \text{ kg} \quad (14)$$

From the values of Eq. (14) the relative uncertainty due to sample mass in tablet, cream, eye ointment and injection was calculated and found to be 2.90×10^{-2} , 2.93×10^{-2} , 2.94×10^{-2} and 2.93×10^{-2} , respectively.

3.3.5. Uncertainty due to recovery of method

Results of recovery were evaluated as percentage recovery from sample matrix of representative spiking. The value of recovery was obtained from validation of method as discussed earlier. When a 'spike' is used to estimate recovery, the recovery of analyte from the sample may differ from recovery of spike, so an uncertainty needs to be evaluated. It was evaluated as Eq. (2) and $U(C_{spike})$ is calculated by using Eq. (15) and results of uncertainty due to spiking concentration of standard are represented in Table 7.

$$U(C_{spike}) = C_{spike} \times \sqrt{\frac{U(C_{bal})^2}{(C_{bal})^2} + \frac{U(v)^2}{(v)^2}} \quad (15)$$

Therefore, the standard relative uncertainty of recovery of method was calculated using uncertainty due to mass of acyclovir (from balance), calibration of pipette, calibration of flask and temperature effect, which was found to be 1.97×10^{-7} , 0.0058, 0.0029 and 0.0048, respectively. Combined uncertainty due to these factors was found to be $U(v) = 0.00805$ mL. Now using the Eq. (3) the standard relative uncertainty due to recovery of method was calculated and the results are represented in Table 7 for different formulations.

3.3.6. Uncertainty due to precision

Method validation results show the repeatability for determination of acyclovir in terms of percentage RSD (0.335). This equation can be used directly for calculation of CSU.

$$U(Rep) = \text{RSD} \\ U(Rep) = 0.00335$$

3.3.7. Combined standard uncertainty (CSU)

The values of all the parameters having effect on acyclovir determination are compiled up in Table 7 for tablet, skin cream, eye ointment and injection, respectively.

These values were further used to calculate acyclovir quantity by using Eq. (1) and thus, we obtained a quantity of 4.06×10^{-5} , 4.00×10^{-5} , 4.06×10^{-5} and 4.07×10^{-5} mol/kg for tablet, skin cream, eye ointment and injection, respectively.

The CSU is calculated according to Eq. (16)

$$\frac{u(ACV_{sample})}{ACV_{sample}} = \sqrt{\left(\frac{u(V_{10})}{V_{10}}\right)^2 + \left(\frac{u(C_{10})}{C_{10}}\right)^2 + \left(\frac{u(m_{sample})}{m_{sample}}\right)^2 + \left(\frac{u(R_m)}{R_m}\right)^2 + \left(\frac{u(Rep)}{Rep}\right)^2} \quad (16)$$

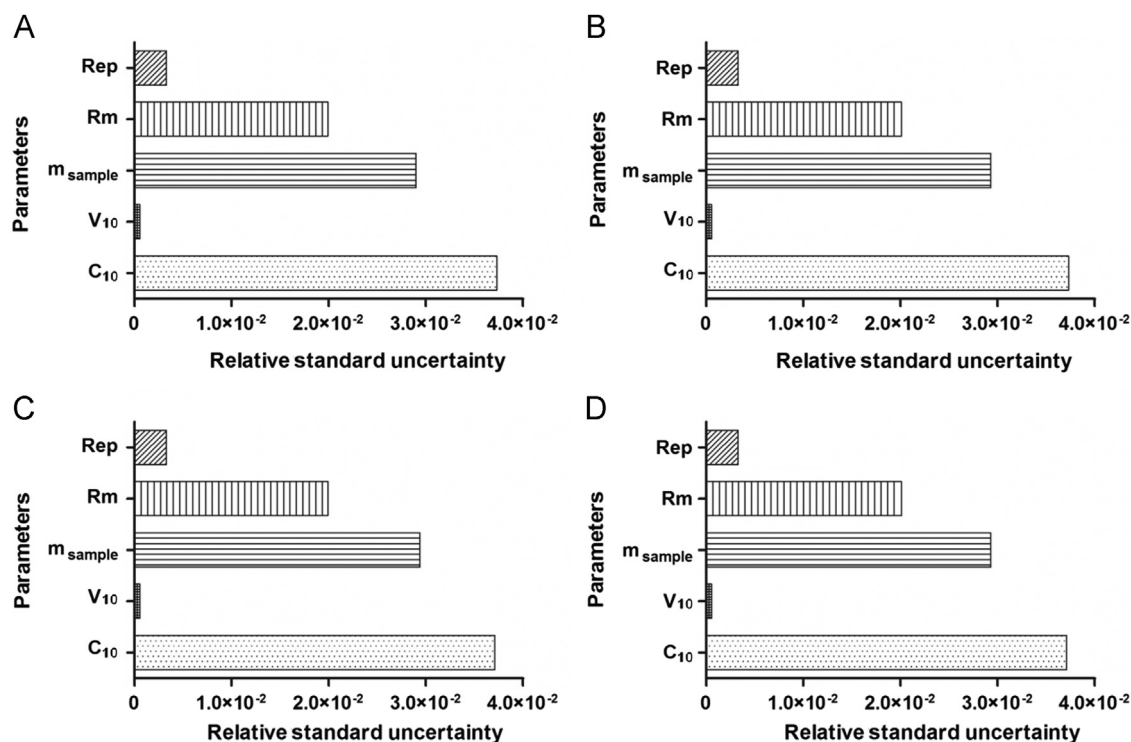


Fig. 5 Uncertainty profile for acyclovir determination in (A) tablets, (B) skin cream, (C) eye ointment, (D) injection representing different components contributing in overall uncertainty and showing the maximum effect due to concentration of sample.

$$u(\text{Acyclovir}_{\text{sample}})^{\text{tablet}} = 2.09 \times 10^{-6} \text{ mol/kg}$$

$$u(\text{Acyclovir}_{\text{sample}})^{\text{skin}} = 2.10 \times 10^{-6} \text{ mol/kg}$$

$$u(\text{Acyclovir}_{\text{sample}})^{\text{ointment}} = 2.06 \times 10^{-6} \text{ mol/kg}$$

$$u(\text{Acyclovir}_{\text{sample}})^{\text{injection}} = 2.09 \times 10^{-6} \text{ mol/kg}$$

3.3.8. Expanded standard uncertainty (EU)

Expanded uncertainty of acyclovir in different sample matrices was obtained by multiplying the combined standard uncertainty by coverage factor, $k=2$, at confidence level of 95%, and the EU ($\text{Acyclovir}_{\text{sample}}$) is as shown

$$\text{EU}(\text{Acyclovir}_{\text{sample}})^{\text{tablet}} = 4.17 \times 10^{-6} \text{ mol/kg}$$

$$\text{EU}(\text{Acyclovir}_{\text{sample}})^{\text{skin}} = 4.20 \times 10^{-6} \text{ mol/kg}$$

$$\text{EU}(\text{Acyclovir}_{\text{sample}})^{\text{ointment}} = 4.12 \times 10^{-6} \text{ mol/kg}$$

$$\text{EU}(\text{Acyclovir}_{\text{sample}})^{\text{injection}} = 4.17 \times 10^{-6} \text{ mol/kg}$$

The contribution of different parameters in uncertainty is shown individually for different sample matrices in Fig. 5.

4. Conclusion

All analytical endeavors generate measurement data and hence, should necessarily employ more or less appropriate statistical techniques and methods of inference, to present and interpret the data. The accurate estimation of variability is challenging. Bayesian approaches offer a different path to the assessment of variability by combining probabilities estimated from detailed study of sub-processes. Developing a new pharmaceutical product requires the designing and testing of manufacturing and measurement processes. The resulting process produces quality products when measurements indicative of product quality are on target

with minimum variance. In the present study, error propagation break up statistical methods are successfully applied. This validation was based on the “total error” approach and it can be seen that the method is suitable for routine analysis of acyclovir in different formulations with minimum errors. In addition, it also illustrates the application of cause–effect analysis in order to estimate the uncertainty in the measuring of acyclovir from different pharmaceutical formulations through UV–vis spectrometry. The estimation of uncertainty components proved to be a good way for the experimental model to obtain contribution of the uncertainty in the analytical result. In the present experiment, concentration of sample is the major contributor towards uncertainty.

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