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Risk outlining and uncertainty contour for quantification of acyclovir using LC-MS: solicitation to *In Vitro* cell line studies

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Abstract

Outlining of risk profile and uncertainty estimation are the two chief and significant strictures that need to be adopted during the development of pharmaceutical process, to ensure reliable results. In this era, the conventional method validation agenda needs to be extemporized so as to certify extraordinary method reliability to measure quality attribute of a drug product. In this research work, risk sketch and expanded uncertainty in the analysis of acyclovir were studied. LC-MS method was validated in our laboratory as per ICH guidelines and risk profile has been outlined including uncertainty estimation using the cause-effect approach. In the course of validation, the calibration model found to be defensible when encountered with Levene's and lack of fit test. The proposed research work evidently demonstrates the application of theoretical concepts of uncertainty and risk profile in the methods used for analysis in drug discovery process.

Keywords:

Cell lines study, LC-MS, risk profile, relative bias, combined standard uncertainty, expanded uncertainty

1. Introduction

In last decades, several analytical techniques has been developed for the analysis of pharmaceutical substances in different matrix. In all of these developed techniques, liquid chromatography-Mass spectrophotometry (LCMS) is the most beautiful gift by the researchers to the analyst as it is the most dependable, having high sensitivity and reproducibility. Nevertheless, in all these techniques we are dealing with data generated from instrument, so we are always at the risk of accurate results and having doubt in our mind about accuracy and reproducibility of results, however these doubts will be very less but are very important as these are concerned with human health care system. Consequently, to overcome these doubts uncertainty estimation is one tool that is rapidly growing and is very effective in the case of doubtful results. In this proposed research work both the newly develop tool uncertainty estimation and LC-MS has been coupled together to have a precise, accurate and strong matrix effect evaluation in the quantification of analyte.

Acyclovir (acv) is one of the utmost used antiviral agents. It is an acyclic guanosine derivative. It has been used in the treatment of genital herpes, herpes simplex and neonatal HSV infection [28, 6, 5]. It has been shown that acyclovir has high solubility and low intestinal permeability and considered as a typical class III drug according to Bio-

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pharmaceutics Classification System (BCS) from the Food and Drug Administration (FDA) of the United States [1, 24]. The therapeutic importance of acyclovir has promoted the development of many analytical methods for its quantitative as well as qualitative estimation. Several methods for the determination of acv has been developed in the past, such as spectrophotometric [23, 10, 21, 26, 3], high-performance liquid chromatography (HPLC) [30, 8, 9, 32, 13, 18, 27, 12, 25, 4], micellar liquid chromatography [20], gas chromatography [19], capillary electrophoresis, and radioimmunoassay [29]. LC-MS/MS is a powerful analytical technique for determining acv due to its shorter chromatographic run time and inherent high sensitivity and selectivity. Although few methods have been developed to quantify the acv but according to our knowledge there is no such method for estimation of acv in caco-2 cell lines and also none of the above published methods has utilized calibration model test, β-expectation tolerance interval, risk profile and uncertainty estimation concepts for the estimation of acv which are critical parameters for today's method validation protocols.

The present research paper describes a LC-MS/MS method for the quantification of acv in formulation as well as in caco-2 cell lines. In this method, the LLOQ, for acv was as low as 50 ng mL^{-1}. As in literature there are some articles which propose the conventional estimation of analytical measurements and uncertainty. However, most of these methods are applied to food samples and a very few methods have been found for pharmaceutical formulations [31, 22, 11, 14, 2]). Therefore, we developed a LC-MS method that overcomes these drawbacks by applying a wide uncertainty estimation and total error estimation approach.

2. Experimental

2.1. Reagents and materials

Acv was procured as gift sample from Nestor Pharmaceutical Pvt. Ltd., India. HPLC-grade methanol was supplied by Rankem and formic acid was supplied by fluka. Water was purified by a Direct-Q ultrapure water system (Millipore, Bedford, MA, USA). Mobile phase used in HPLC was filtered using a 0.45-mm membrane filter Commercial formulations of acyclovir were purchased from local drug store. Caco-2 cell line was procured from NCCS Pune.

2.2. Instrumentation

Chromatographic study was performed using ekspertTM ultraLC with ekspertTM ultraLC 100 pump system (eksigent-AB Sciex, USA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, USA), located at Dr. Vikram Sarabhai Science Center, Faculty of Science, The M.S. University of Baroda, Vadodara, Gujarat, INDIA. 20 µL of each sample was injected. The autosampler system (ekspertTM ultraLC 100 XL, eksigent-AB Sciex, USA) was tempered to 8°C equipped with column oven (ekspertTM ultraLC 100, eksigent-AB Sciex, USA) fixed at 40°C. Chromatographic elution of analyte was achieved using a Phenomenax C18 5µm (250*4.6) mm column at a flow rate of 0.5 mL min^{-1} for having run time 8 mins. The isocratic composition of eluent a (water with 0.1% formic acid) and eluent b (methanol) was in 60:40 % v/v.

2.3. LC-MS Conditions

Analysis was conducted using 3200 QTRAP mass spectrometer (AB Sciex, USA) equipped with electro spray ionization (ESI) source. The mass spectrometer was operated in the positive ion mode with a potential of 5.5 kV applied on the electro spray ionization needle. The ionization source temperature was 600 °C. Acv was identified and quantified using Multiple Reaction Monitoring (MRM) mode. The curtain gas (CUR) was at 25.0 psi,

the nebulizer source gas 1 at 50.0 psi and the turbo ion source gas 2 at 50.0 psi was utilized. The optimized Declustering potential and entrance potential were 60.0 V and 5.6 V respectively. Acv fragmentation was achieved by collisionally activated dissociation (CAD) with nitrogen gas. The collision gas pressure was fixed at 2.0 psi for MRM quantitation. The collision energy 22.0 V and collision cell exit potential 3.0 V were optimized. Dwell time 200 ms was used. The product ion at m/z 226.00 was selected.

2.4 Preparation of Stock Solutions, Calibration and Validation Standards

An accurately weighed amount of acv was transferred into a 10 mL calibrated flask and dissolved in 5 mL of mobile phase. The resulting solution were completed to the mark with mobile phase obtaining stock standard solution containing 1000 μg mL^{-1}. Stock solution were then further diluted with mobile phase to obtain the working standard solutions at concentrations over the range of 50–1600 ng mL^{-1}. Six calibration standards were prepared at concentrations of 50, 100, 200, 400, 800 and 1600 ng/mL. Validation standards were similarly prepared at levels of 100, 200, 400 and 800 ng mL^{-1}.

2.5 Caco-2 cell line and formulation sample preparation

The samples for the acv permeation studies were collected at different time points from the basolateral side from the transwell plates. The collected samples were filtered and diluted with the mobile phase. The prepared samples with unknown concentrations has been injected. The formulation samples were prepared by crushing twenty tablets up to fine powder and then an accurately weighed quantity of the powdered tablet contents equivalent to 10 mg of the active ingredient was transferred into a 10 mL calibrated flask and dissolved in about 6 mL of mobile phase. The contents of the flask were swirled, sonicated up to 9 minutes and then volume of the flask was made up with mobile phase. The mixture was mixed well, filtered and first portion of the filtrate was rejected. The prepared solution was diluted quantitatively with the mobile phase to obtain a suitable concentration for analysis.

2.6 Validation Procedures using total error approach

The present method was validated as per ICH guidelines (ICH 1994 [16]; ICH 1996[15]) and ISO guidelines which were grounded upon “total error” approach (ISO-IEC 1999[17]). In this approach “total error” was estimated by merging the systemic error and random error to recognize the difference between observed and true value. In the proposed method sensitivity of the method and effect of sample matrix were also studied. The selectivity of the studied method was investigated by comparing chromatograms of blank cell lines without acv, blank mobile phase and sample of cell lines with acv and sample of formulation as shown in (Fig. 1). Response function in proposed method four sets of calibration curve were plotted between area and different concentrations of acv and on these four different series regression analysis was performed and series with best coefficient of determination was selected and the selected series has been further diagnosed by Lack of Fit (LOF) test and standard residual plot. Trueness of calibration curve was calculated by back calculation of concentrations to justify the calibration line. The results of trueness were expressed in terms of absolute and relative bias. The recovery study which is the most critical parameter in method validation requires an extra precautions during study and interpretation of recovery results. Therefore, the results of accuracy studies were interpreted and represented in the β-expectation tolerance limits. In addition to these parameters, risk profile has also been studied to know the future application of the method. Limit of detection and quantification represents the sensitivity of the method which has been calculated as per ICH guidelines. Subsequently confirmation of method fitness for the estimation of acv in different matrix was carried out by analyzing market formulation and cell line samples.

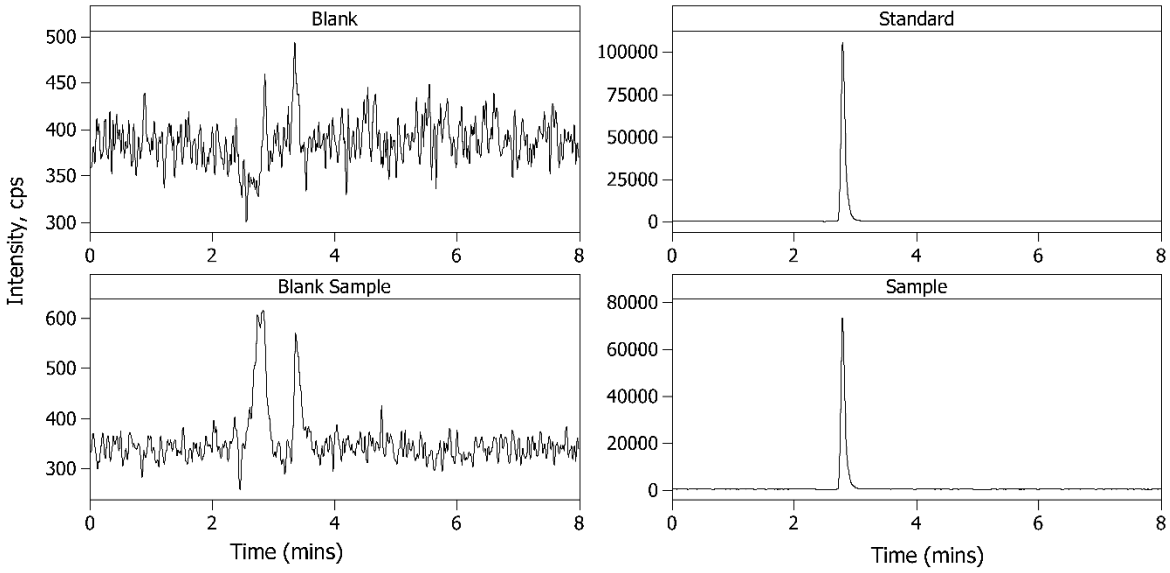

Fig.1: Chromatogram of blank, blank sample cell line, standard and sample

2.7. Uncertainty Estimation

2.7.1. Cause-effect diagram

Even though estimation method was validated as per guidelines but still doubt was there in results as during the validation of method small influences which can affect the results has not been studied, such as error during sample weighing, discharge of volumetric flask etc. Therefore, to overwhelm such doubts during result collation were clarified by estimation of uncertainty in results obtained from validation. The protocol for uncertainty estimation starts with identification of sources of uncertainty. The best way of listing uncertainty sources is to use the cause-effect diagram plan, as it outlines the sources connection to each other demonstrating their impact on the result. Thus a cause-effect diagram was assembled as presented in (Fig. 2). The parameters taken in consideration were volume of volumetric flask V_{10}, concentration of analyte C_{10}, and mass of sample, recovery of method R_m and precision of method. This diagram also help in resolving any repeatability of components in uncertainty. The parameters comes in consideration after constructing cause-effect diagram were illustrated in (Equation 1).

$$ACV_{sample} = C_{10}V_{10}10^{-3}/m_{sample}\, R_m \qquad (1)$$

Where, $acv_{sample,}$ acv quantity (mol/kg); $C_{10,}$ acyclovir concentration in 10 mL volumetric flask (M); V_{10}, volume of 10 mL volumetric flask (mL); m_{sample}, acyclovir sample mass taken (kg); Rm, Recovery of method.

These identified sources were quantified and their discrete effect of on inclusive uncertainty was calculated and assembled as CSU and EU.

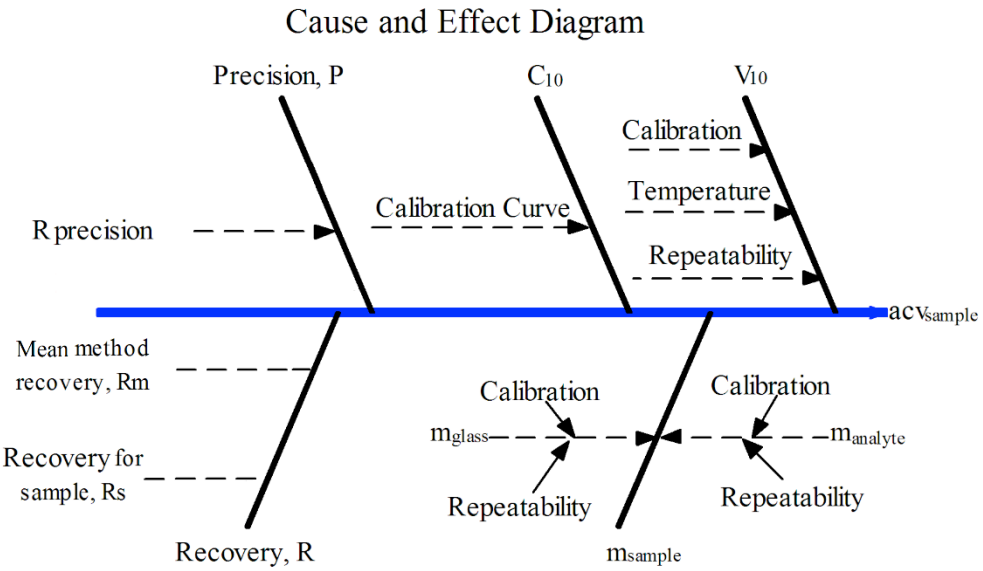

Fig.2: Cause and effect diagram to identify the sources of uncertainty

2.7.2 Individual parameters showing effect on overall uncertainty

2.7.2.1 Liberation of acv solution from volumetric flask

The uncertainty due to liberation of volumetric flask was evaluated by performing experiment involving filling up and weighing of 10 mL volumetric flask with standard acv solution for 10 times.

2.7.2.2 Acv mass (m_{sample})

Difference between weighing glass with and without the acv sample provide the acv sample mass.

2.7.2.3 Concentration of acv, C_{10}

The uncertainty in concentration of acv obtained from calibration curve is expressed as uncertainty due to concentration C_{10}. This is estimated using (Equation 2).

$$U(c) = \frac{Sr}{b}\sqrt{\frac{1}{n}+\frac{1}{p}+\frac{(c-\bar{c})^2}{Sxx}} \qquad (2)$$

Where: $S_r = \frac{\sqrt{\sum_{j=1}^{n}[Yj-(bxi+a)}}{n-2}$

$S_{xx} = \sum(Ci-\bar{c})^2$

S_r, standard deviation of residual; n, number of measurements used for calibration curve; p, number of measurements used to obtain concentration of sample; c, acyclovir concentration in sample (M); $\bar{c}$, average of standard solution (M); Y_j, response obtained from the measurement; j, index for number of measurements made in order to obtain the calibration curve; i, index for number of solution for calibration; b, slope of calibration curve (L mol^{-1}); a, calibration curve intercept;

2.7.2.4 Recovery of method

Uncertainty associated with recovery of method was evaluated using (Equation 3) and it depends upon spiked and recovered concentration of standard in sample matrix.

$$U(Rm) = Rm \times \sqrt{\left(\frac{Sobs^2}{n \times Cobs^2}\right) + \left(\frac{U(Cspike)}{Cspike}\right)^2} \quad (3)$$

Where C_{obs}, mean of concentration observed from replicate analysis of spiked sample; C_{spike}, nominal concentration of acv in spiked sample. S_{obs}, means standard deviation of results from the replicate analyses of spiked sample; n, number of replicates; U (C_{spike}), standard uncertainty in concentration of spiked sample.

3. Results and Discussions

3.1 Method Development and Optimization

Optimization of the chromatographic conditions is the most critical step having a very specific aim to achieve symmetrical peak shapes with short chromatographic analysis time also having high sensitivity and selectivity. During the optimization higher responding signals and less interference of sample matrix endogenous substances were observed in negative ion mode than positive ion mode by comparing the obtained chromatograms. Thus, negative ion mode was chosen. Ion transitions at m/z 226.0 for acv were selected for quantification. The CE, DP, CXP, and EP for acv were optimized to obtain the greater intensity of the target ion pairs. The CE of 40 and 25, DP of 150 and 100, CXP of 10, and 13 and EP of 11 and 11 for acv were adopted, respectively.

3.2 Validation parameters

In the proposed method calibration curves from the response of different concentration were prepared using linear regression model. The four different sets were prepared for response function studies with range of acv from 50-1600 ng/mL, from their regression analysis studies series 3, shows the best results with coefficient of determination (r^2) 0.9997, so this series was selected for further computation for validation and sample analysis. Moreover, the selected series and regression model was diagnosed and confirmed using Lack of Fit (LOF) test. The p-values were calculated and found to be greater than 0.05, as illustrated in Table 1 and further to demonstrate that no outliers were found in calibration curve standard residual plot were also plotted as represented in (Fig. 3)**.** As the model was established, now in order to authenticate the regression equation back calculation was done and linear plot using absolute β-expectation limit was constructed between nominal and back calculated concentration which showing the r^2 0.9998 and confirming the authenticity of regression equation. Trueness of method was justified by calculation of %age relative bias which was found to be limited between [-0.03524% -- 0.3887%] as illustrated in Table 2 from which it has been concluded that trueness of method is adequate. The method precision and reproducibility was authenticated by results obtained from precision studies which were found to be < 2% in terms of RSD for both repeatability and intermediate levels as illustrated with 95% confidence upper limit in Table 3. After the conformation of accuracy of all the parameters related to system and developed method, sample matrixes was incorporated in validation process which includes recovery studies. Recovery studies were carried out using standard addition method in sample matrixes. These recovery studies receipts into account total error of test results and is represented by the β-expectation tolerance limits. The results of accuracy studies has been illustrated in Table 4. The β-expectation tolerance limits was also found to be in the acceptance as accuracy profile illustrated in (Fig. 4). Further, these recovery studies of the method was justified by plotting risk profile keeping maximum risk level at 5.0% from which it was concluded that risk of outliers are within limits and in future analysis of the samples using this developed and validated method will fall within range. The results of LOD show that this method is sensitive enough to analyze the marketed formulations and cell line samples, LOD was found to be 0.189 ng mL^{-1} resp.

Table 1. Results of LOF for linear regression model

		SS	df	MS	Fcalc	Fcrit,95%	p-value
Lack of Fit test	LOF Error	7791	8	973.9	1.058	2.849	0.4486
	Pure Error	1.1043×10^4	12	920.3			

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

Nominal concentration (ng/mL)	Back calculated concentration (ng/mL)	Absolute bias (ng/mL)	Relative bias (%)
50.00	50.19	0.1943	0.3887
100.00	99.82	-0.1753	-0.1753
200.00	199.5	-0.5356	-0.2678
400.00	399.6	-0.3630	-0.0908
800.00	801.4	1.443	0.1804
1600.00	1599	-0.5638	0.0352

Table 3: Results of relative and absolute intermediate precision and repeatability in terms of (%RSD)

	Relative intermediate precision and repeatability				Absolute intermediate precision and repeatability	
			95% Upper Confidence Limit			
Nominal Conc (ng/mL)	Rep* (%RSD)	Intermediate precision (%RSD)	Rep* (SD) (ng/mL)	Intermediate Precision (SD) (ng/mL)	Rep* (SD) (ng/mL)	Intermediate precision* (SD)(ng/mL)
50.00	0.0078	0.1088	0.0172	0.8662	0.0039	0.0544
100.00	0.7499	0.8267	2.1901	3.2693	0.7356	0.7259
200.00	0.4559	0.9245	0.3919	3.7574	0.9117	0.9117
400.00	0.1527	0.3867	2.1690	3.8732	0.6108	0.9916
800.00	0.1788	0.1239	1.9862	6.4502	1.4304	1.849
1600.0	0.1032	0.3249	0.2609	8.1545	1.6524	0.8532

Table 4. Result of method accuracy in terms of relative beta-expectation tolerance limit and risk assessment obtained by selected regression model in matrix

	Concentration Level (%)	Concentration (ng/mL)	Beta-expectation tolerance limits (ng/mL)	Relative Beta-expectation tolerance limits (%)	Risk[1] (%)
Tablet	80.0	80.00	[78.29 , 81.71]	[-2.138 , 2.140]	0.1998
	100.0	100.00	[98.55 , 101.4]	[-1.446 , 1.448]	0.0349
	120.0	120.00	[117.9 , 122.1]	[-1.723 , 1.724]	0.07719

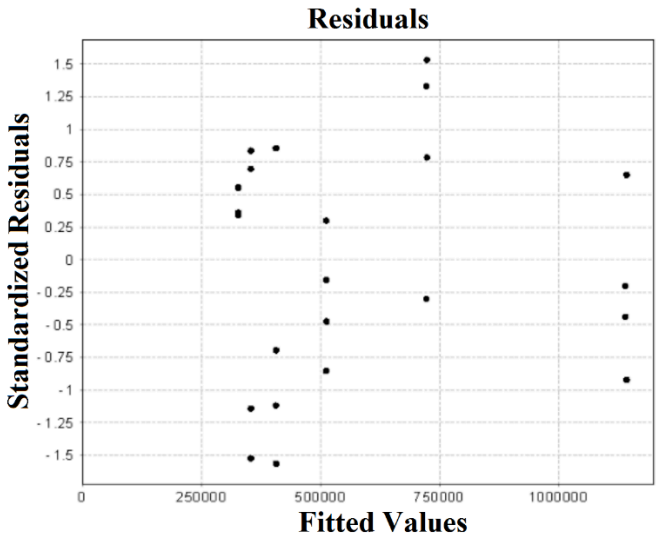

Fig.3: Standard residual plot of representing absence of outliers at different concentration levels.

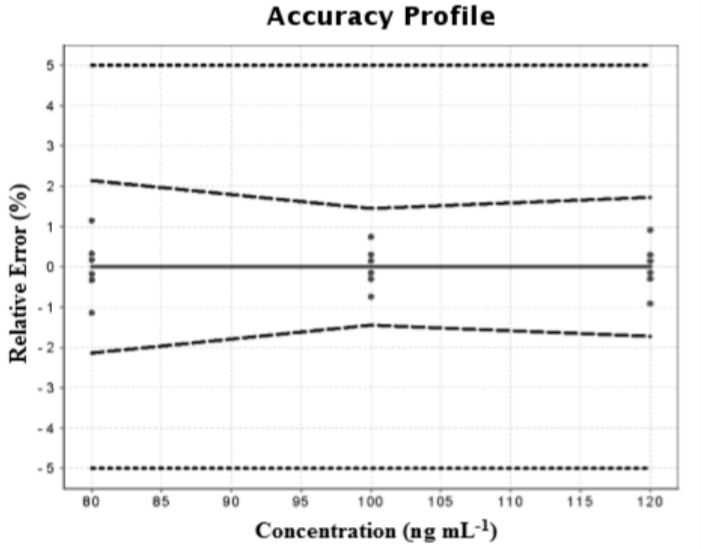

Fig.4: Accuracy profile of acyclovir obtained after application of linear regression using calibration standards prepared with the matrix. The plain line is the relative bias, the dashed lines are the 95% β-expectation tolerance limits and the dotted curves represent the acceptance limits (±5%). The dots represent the relative back-calculated concentrations of the validation standards**.**

3.3 Application of the developed method to cell line and formulation

3.3.1 Analysis of Formulation

It is evident from the aforementioned results that proposed method gave satisfactory results with the acv in bulk drug. Thus dosage forms were subjected to analysis for their contents of active drug material by the proposed method. The percentage purity for tablet were found to be 100.15 %. It is evident from the above mentioned results that proposed method is applicable to the analysis of drugs in its bulk drug as well as dosage forms with comparable analytical performance.

3.3.2 Analysis of cell line samples for permeation studies

The results obtained from the analysis of formulation were found to be satisfactory so, this method has been applies to the cell line samples for permeation studies. The results obtained from the cell line studies are represented in (Fig. 5).

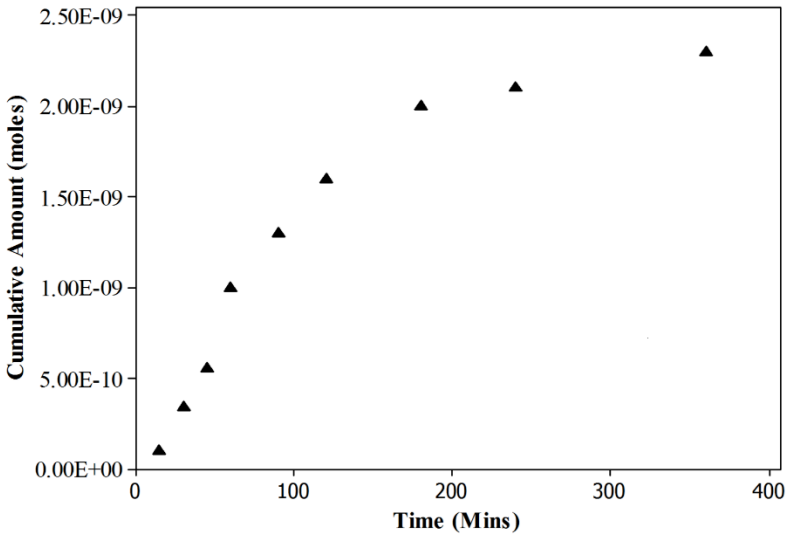

Fig.5: Plot of concentration of acyclovir obtained from cell line samples vs time.

3.3.3 Measurement of uncertainty

Once uncertainty sources has been identified, they were evaluated and their magnitude was determined. In order to assure the traceability for uncertainty results all the computations were done in International System of Units as concentration in M and weight in kg.

3.3.3.1 Uncertainty of volumetric flask

The uncertainty due to volumetric flask is mainly influenced by the three parameters i.e. calibration of the volumetric flask at the time of manufacturing, repeatability and temperature.

3.3.3.1.1 Calibration of volumetric flask

Deviance from nominal volume of 10 mL volumetric flask is ± 0.006 mL (at 27°C) as given by manufacturer. Standard value of uncertainty can be calculated with triangular distribution. So, uncertainty related to the liberation of volume by volumetric flask (u (V_{10}cal)) is 0.0024.

3.3.3.1.2 Repeatability, u (V_{10}rep)

In experiment repeatedly weighing and filling of volumetric flask standard uncertainty established was 0.0016 mL.

3.3.3.1.3 Temperature

The manufacturer has calibrated volumetric flask at time of manufacturing at temperature of 27°C, while temperature at laboratory varied with $\Delta t = \pm 4$ °C. This difference can be 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, λ, of water is 2.1×10^{-4} /°C. Uncertainty for 10 mL volumetric flask ΔV_{10} was calculated by (Equation 4).

$$\Delta V_{10} = V_{10} \times \gamma \times \Delta t \qquad (4)$$

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 uncertainty for volumetric flask of 10 mL is 0.0084 mL, standard uncertainty due to temperature on liberation of volumetric flask was found to be 0.0048 mL.

3.3.3.2 Uncertainty associated with the sample mass m_{sample}

Sample mass has three types of uncertainty sources sensitivity, linearity, and repeatability. Mass of the sample was expressed in kg to convince traceability of results.

3.3.3.2.1 Sensitivity

The difference in weighed mass was in very less range and it was measured on the same weighing balance. Thus uncertainty due to sensitivity of balance can be neglected.

3.3.3.2.2 Linearity

A rectangular distribution was assumed to convert contribution of linearity. It was calculated as (Equation 5).

$$u = \frac{1.06 \times 10^{-7}}{\sqrt{3}} = 6.12 \times 10^{-8}\text{Kg} \qquad (5)$$

3.3.3.2.3 Repeatability

Uncertainty associated with repeatability is found to be 2.08 x 10^{-7}kg.

3.3.3.2.4 Computation of relative uncertainty due to sample mass

Using the uncertainty due to linearity and repeatability the uncertainty due to sample mass u (m_{sample}) was calculated using (Equation 6).

$$u(m_{sample}) = \sqrt{2 \times (6.12 \times 10^{-8})^2 + (2.08 \times 10^{-7})^2} = 2.25 \times 10^{-7}\ \text{Kg} \qquad (6)$$

3.3.3.3 Uncertainty associated with Concentration, (C_{10})

Analytical responses were collected after each injection of standard solution of different concentrations. These responses were used to construct calibration curve. Regression equation of calibration curve was identified such as, slope 8.66 x 10^{13} and intercept 342014.34. Uncertainty involved in the construction of calibration curve was estimated by injecting 6 different concentration solutions each measured three times and sample solution was measured ten times from which Sr and Sxx values were computed as shown in (Equation 7 and 8), which were further used to calculate standard relative uncertainty, due to concentration.

$$Sxx = 3.47 \times 10^{-17} \qquad (7)$$

$$Sr = 273.03 \qquad (8)$$

3.3.3.4 Uncertainty due to recovery of method

Results of recovery are evaluated as percentage recovery from sample matrix after spiking a known amount. When term ‘spike’ is used to estimate recovery, the recovery of analyte from the sample may differ from recovery of spike so that an uncertainty needs to be evaluated. Uncertainty due to spiking is found to be 8.76 x 10^{-10}. Standard relative uncertainty of method recovery 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 1.98

x 10^{-5}, 0.0052, 0.0029 and 0.0048 respectively. Combined uncertainty due to these factors were found to be U (Rmf) = 1.99.

3.3.3.5 Uncertainty due to precision

Method validation results show the repeatability for determination of acyclovir in terms of % age RSD (0.6793). This equation can be used directly for calculation of CSU.

$$U(Rep) = RSD$$

$$U(Rep) = 0.6793$$

3.3.3.6 Combined standard uncertainty (CSU)

The values of all the parameters having effect on acyclovir determination, these are compiled up in Table 5. These values of parameters were further used to calculate acyclovir quantity by using Equation 1 and thus, we obtained a quantity of 4.17 x 10^{-7}, mol/kg.

Table 5: Summary of contribution to the measurement uncertainty for determination of of acyclovir through UV-Vis Spectrometer

Formulation	Parameter	Volume, V_{10} (mL)	Sample conc. C_{10} (M)	Mass sample, m_{sample} (kg)	Recovery method	Repeatability
Tablet	Value	10	4.43 x 10^{-10}	1.06 x 10^{-5}	100.20 x 10^{-2}	-----
	Standard uncertainty, u(x)	3.16 x 10^{-5}	1.60 x 10^{-12}	2.25 x 10^{-7}	1.99	6.79 x 10^{-3}
	RSU*, u(x)/x	3.16 x 10^{-6}	3.60 x 10^{-3}	2.13 x 10^{-2}	1.98 x 10^{-2}	6.79 x 10^{-3}

3.3.3.7. Expanded Standard uncertainty (EU)

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

$$EU\ (Acyclovir_{sample})\ tab = 2.51 \times 10^{-8}\ mol/kg$$

The contribution of different parameters in uncertainty is shown individually for sample matrix has been illustrated in Fig. 6.

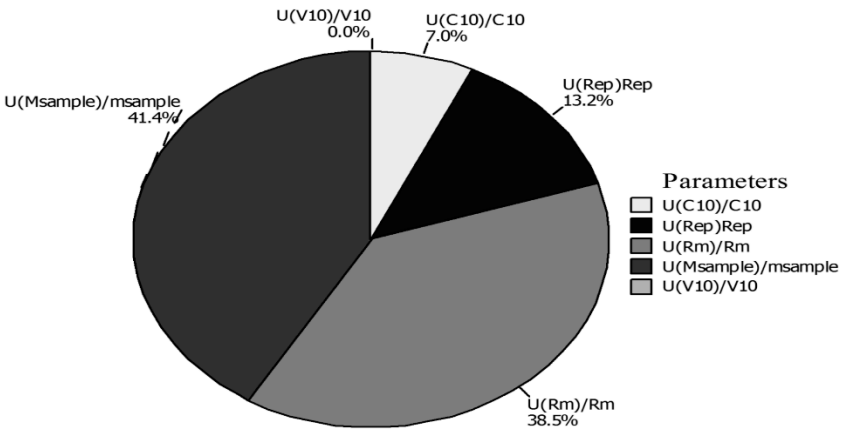

Fig. 1: Uncertainty profile representing different components contributing in overall uncertainty

4. Conclusions

All analytical endeavors generate data and hence, should be necessarily employ an appropriate statistical techniques to interpret the data. The estimation of inconsistency is challenging. Several statistical approaches offers different path for the assessment of variability by combining probabilities estimated from detailed study of sub-processes. In the present study, error propagation break up statistical methods are successfully applied. In this research work validation was based on the "total error" approach and it can be seen that the method is suited for routine analysis of acyclovir in different formulations and cell lines studies with minimum errors. This work also illustrates the application of cause-effect analysis in order to estimate the uncertainty in the measuring of acyclovir from pharmaceutical formulations and in-vitro studies through LC-MS. 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.

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