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ACCURACY TARGETED HPLC-UV METHOD FOR THE ESTIMATION OF AMLODIPINE AND INDAPAMIDE

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ABSTRACT

Accuracy of an analytical method is ensured by appropriate method validation process. Guidelines laid by the regulatory bodies can be a general framework to assess the validity of a method. Since these guidelines provide marginal accuracy of analytical results, this study was aimed to test a recently evolved strategy that may render analytical method validation more accurate and trustworthy. The concentration range in which reliable analytical results can be obtained is proposed by a Bayesian probability study. Such study renders perfect information about the concentration range of the analyte which may produce accurate analytical results. In order to ensure the applicability of this approach, it was applied for the validation of a HPLC–UV assay method developed for the quantification of amlodipine and indapamide in human plasma. A comparative study between the newer approach and the standard method validation proved that the application of Bayesian analysis at the end of the validation process can produce significant improvement in the analytical results.

Key Words: Reliability, Bayesian, Accuracy, Analysis, validation, statistics.

INTRODUCTION

Results accuracy is an important aspect of analytical method validation. The need to check the accuracy of an analytical method is done by analysts in the pharmaceutical industry on an almost daily basis, because adequately validated and reliable methods are a necessity for approvable regulatory filings. Recent developments in analytical instrumentation have resulted in enormous progress in method development. The use of chemometric tools enables the development and optimization

of analytical procedures in laboratories and industries worldwide[1]. When advancements in method development are remarkable, the need for innovations in method validation is also important to achieve genuine analytical results. The International Conference on Harmonization (ICH) has issued guidelines for analytical method development and validation. This shows the interest of regulatory agencies for method validation is obvious[2].

Accuracy is a continuous process and the aim is to ensure confidence in the analytical data throughout the analytical method development. It should be designed by the developer or user to ensure repeatability of the method. Methods should be reproducible when used by the other analysts, on other equivalent equipment, on other days or premises and throughout the life of the drug product. Hence, data that are generated for acceptance, release, stability, or pharmacokinetics will only be trustworthy if the methods used to generate the data are reliable[3].

For over two decades, classical validation parameters were widely used in method validation. In the recent past, implementation of computers and software has created a tremendous change in analytical method validation. However, this is not enough and a further step needs to be performed by means of statistical treatment to enhance the accuracy of the experimental data. Hence, enhancement in method accuracy is highly significant in analytical methods. This study explores the application of a novel strategy that may transform the analytical method more reliable, straightforward and trustworthy.

2 Results accuracy of the analytical method

Results accuracy is an important criterion at the end of an analytical method validation. It is defined as the probability of an analytical method to provide analytical results (X) within predefined acceptance limits (±) around their reference concentration values (T) over a defined concentration range and under given experimental conditions[4]. This research proposes a Bayesian analysis, which can resolve the issue of results accuracy to a greater extent.

2.1 Basic principles of Bayesian probability analysis.

Generally, Bayesian analysis can be executed by the following four steps.

Step 1: A probability model is formulated initially for the data.

Step 2: The prior distribution that quantifies the uncertainty in the values of the unknown model parameters is decided before the data are observed (the prior distribution).

Step 3: The likelihood function is constructed based on the observed data and the probability model formulated in the first step. The likelihood and the prior distribution are then combined to determine the posterior distribution, which quantifies the uncertainty in the values of the unknown model parameters.

Step 4: The quantities of interest based on the posterior distribution are calculated, after the data are observed (the likelihood function). These quantities constitute statistical outputs, such as point estimates and intervals. The concepts involved in Bayesian analysis are depicted in Fig. 1.

The overall objective of a Bayesian statistical analysis is to obtain the posterior distribution of model parameters. In reality, the posterior distribution is a weighted average between knowledge about the prior distribution and likelihood function. In other words, posterior distribution is proportional to the prior times a likelihood, i.e. Posterior ∝ Prior × Likelihood. From a Bayesian perspective, any kind of inferential queries can be answered through an appropriate analysis of the posterior distribution. Once the posterior distribution has been obtained, one can calculate the point and interval estimates of parameters, prediction inference for future data, and probable assessment of hypotheses[5–15]. The method used in this study uses the fundamental design of analytical method validation and assumes the following equation:

$$X_{ijk} = \beta_0+\beta_1\mu_{T},i + \mu_0,j + \mu_1,j\mu_{T},i +\varepsilon_{ijk} \quad (1)$$

Where the subscripts i represent the I concentration levels of the validation standards, j for the J number of series or runs and k for the K number of replicates per run. μT,i is the ith concentration level of the validation standard and is regarded as a reference or conventional true value. In analytical practice β_0 and β_1 are the constant bias and proportional bias, respectively. Additionally, 0, j, 1, j are the random effects of the jth runs and gijk is the residual error coming from a normal distribution of variance 2i.

Finally, by specifying the acceptance limits, the accuracy probability as a function of the reference concentration T,I is obtained. When the Bayesian model specification is completed, Markov Chain Monte Carlo (MCMC) sampling can be executed, to obtain the posterior distribution of each parameter. MCMC methods are a class of algorithms for sampling from probability distributions based on constructing a Markov chain that uses the previous sample values to randomly generate the next sample value. From the posterior distributions obtained from MCMC sampling, the predictive distribution of the reliability probability can be obtained for any concentration level T,i. From this the posterior accuracy probability of results lying inside the acceptance limits [±λ] can be obtained analytically[16]. Finally, a graph representing the accuracy of the results obtained by a quantitative analytical method over the whole concentration range studied can be obtained, and the concentration range over which the method is sufficiently reliable can be determined by comparing the posterior accuracy probability to a minimum accuracy value (min) for, e.g. 95% for a bio analytical study.

MATERIALS AND METHODS

Reagents and chemicals

Amlodipine (AM), indapamide (IM) and domperidone (DM) were obtained from Roxaane Research Pvt. Ltd (Chennai, India). Acetonitrile, pentanesulfonic acid, phosphate buffer, ether, and n-hexane were procured form Qualigens Fine Chemicals (Mumbai, India).

Apparatus

The HPLC system consisted of a Shimadzu Prominence HPLC (Kyoto, Japan) and chromatography was performed on a Phenomenex $C1_8$ column, 5m, 250 × 4.6 mm id (Amsterdam, The Netherlands) operated at ambient temperature. Evaporation of the Plasma sample was effected by a TurboVap low-volume evaporator (Uppsala, Sweden).

Chromatography

The mobile phase used was a combination of ammonium acetate buffer (pH 5.0, solvent A) and acetonitrile (solvent B) (53:47, v/v). The flow rate was 1.2mL/min and the wavelength of detection was set at 220 nm.

Preparation of standard solutions

A stock solution of AM and IM, and internal standard DM was prepared in water (1 mg/mL) and stored at 4±1ºC. Working standards were prepared freshly on each day by appropriate dilution of the stock solution.

Extraction of plasma samples

Calibration standards and quality-control (QC) samples were added in a quantity of 25–250µL of plasma in an Eppendorf tube and mixed well. Phosphate buffer (0.01 M) was added in a quantity of 100 µL and centrifuged for 30s; then it was extracted with 300µL of ether. The organic layer was separated from the plasma and evaporated in a low-volume evaporator and reconstituted with 200µL of mobile phase. It was washed with 500µL of n-hexane and a volume of 25µL was injected into the HPLC system.

Preparation of calibration standards and validation standards

Aliquots not exceeding 20µg/ml to prepare calibration standards resulting in 150, 300, 600, 1200 and 2400 ng/ml of AM and 50, 100, 200, 400 and 800ng/ml of IM and 500ng/ml each of RT as the internal standard (IS). Three sets of calibration curves were prepared in the matrix and analyzed during the validation process in triplicate. The linear regression equation assuming y=mx+c was obtained by feeding the values of signals against concentrations in GraphPad Prism 5.01 software. A stock solution of each analyte was prepared at the same concentration explained above. Similarly, validation standards were prepared in human plasma to contain 300, 900 and 1800 ng/ml of AM and 150, 300 and 450 ng/ml of IM and a quantity of 500 µg/ml each of RT as the internal standard (IS) and extracted using the procedure mentioned in Section 3.2.The validation standards were analyzed over three days with three replicates.

Validation of the developed HPLC–UV method

Validation process was carried out according to ICH guidelines: validation of analytical procedures: text and methodology Q2 (R1)[17]. The general validation criteria were obtained using e-noval V3.0 software (Arlenda, Liege, Belgium).

Selectivity

The selectivity of the assay method was assessed by evaluating potential interference from endogenous compounds. Six randomly selected blank plasma samples were analyzed under the optimized chromatographic conditions described. The separation of AM, IM, and IS and probable endogenous compounds from plasma were checked by comparing the chromatograms of QC samples with that of drug-free blank plasma, in order to evaluate interference of the retention times of AM, IM and IS. The samples were extracted and analyzed as described in Section 3.5. The peak and the retention time for each drug under the chromatographic conditions of the AM, IM, and IS assay was recorded to assess whether the peak for plasma endogenous components interferes with the peaks of AM, IM and the IS.

Linearity

Peak area ratios (drug/IS) were plotted against the corresponding AM and IM nominal concentrations. Least-squares regression analysis of the linear data was performed using the equation y=mx+c; where m and c are the slope and intercept of the calibration curve. The linearity was evaluated from the slope, intercept, and correlation coefficient (r^2) of each curve. The calibration standards were aliquoted and assayed on three successive days to

demonstrate the linearity of the method by the OLS method. The assay was corrected for blank human plasma drug levels by deducting the response of endogenously occurring drugs in each individual pool from the corresponding total response of the drugs in the spiked plasma.

Lower limit of quantization (LLOQ)

The LLOQ was regarded as the lowest analyte concentration that can be determined with a precision of<20% expressed by RSD that produces an S/N of 1:3 between sample and blank.

Precision and accuracy

The precision and accuracy of the method were assessed at three concentration levels (QC 200, 400, 800 µg/mL of AM and IM). Three replicate samples were prepared and analyzed on the same day for intraday and continuous days (n=4) accuracy and precision according to ICH guidelines. The assay precision was expressed as RSD and accuracy was calculated in terms of bias. The intra- and inter-day precision were required to be <15%, and the accuracy to be within ±15%.

Recovery (extraction efficiency)

The recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the pure equivalent of authentic standard. The extraction recovery of AM, IM, and IS in plasma was measured at three QC concentration levels (QC 200, 400, 800 ng/mL of CF and OZ).

Dilution integrity

Dilution integrity was assessed by assaying six replicates of the QC samples spiked with approximately two times of 90% concentration of the upper level of quantification and diluted by a factor of two and four prior to extraction. The samples were processed and analyzed against freshly spiked calibration standards.

Stability

Post preparation stability was assessed by reanalyzing QC samples after 24 h in an autosampler at 4C. Bench-top stability was assessed by reanalyzing the QC samples after 6 h at room temperature. The analyte was considered to be stable in plasma when 85–115% of the initial concentration measured emerges back in the sample.

Evaluation of accuracy of analytical results

The concentration range selected from the above procedure was then treated further to obtain the reliable range for future analysis by Bayesian analysis. Bayesian analysis was applied, by fixing the acceptance limits within which the results must fall. The acceptance limit was set at a maximum of ±20% around the reference concentration values (T,i) of the validation standards. The minimum accuracy criterion (min) was set at 90. Having set these limits, the profile of accuracy can be framed out for the analytes of interest. The Bayesian accuracy profile was performed with SAS/STAT9.1.3 version (SAS Institute, Cary, NC, USA) pack-age for MCMC sampling. The Bayesian model specification has been devised for the data obtained from method validation. MCMC sampling has been executed, using statistical software SAS/STAT 9.1.3, to obtain the posterior distribution of each parameter. The predictive distribution of the accuracy probability for any true concentration level is then obtained following a suitable algorithm[18–26]. From the posterior distribution explained above, the predictive distribution of the accuracy probability for any concentration level (T,i) can be ascertained.

Bayesian simulation enables estimation of any model that can be determined by the maximum likelihood of occurrence. Any-thing about a random variable x can be learned by sampling many times from f(x),

the probability density function of x. From the posterior distribution of the parameters obtained with the MCMC sampling, the posterior accuracy probability of results following the normal distribution lying inside the acceptance limits can be obtained analytically. Finally, the results accuracy of the analytical method developed for the entire concentration range investigated can be ascertained by a graph obtained by the procedure explained in Section 2.3. Following the determination of the valid concentration range for each component present in the matrix through Bayesian analysis, plasma samples of CF and OF were pre-pared at two concentration levels; one to fall within the Bayesian accuracy range and the other to fall out of the Bayesian range to assess the legitimacy of the method. The precision values (percentage CV) obtained for the two ranges of sample were then compared.

Comparison of the new validation protocol with the usual validation protocol

Comparison between the new validation protocol (that includes Bayesian analysis) and usual validation protocol has been carried out in terms of percentage relative error to study the effect. Information about percentage relative error can be obtained through accuracy profiles for CF and OF conveniently.

Construction of accuracy profile

Calibration and validation standards were measured using usual validation protocol and the newer protocol on three days with two replicates per day for the calibration standards and three replicates per day for the validation standards. Accuracy profiles were generated using the results obtained from the two validation protocols and the mean of percentage relative error values obtained from the two schedules were compared directly for any significant difference[27-26].

RESULTS AND DISCUSSION

Method validation results

Selectivity

The selectivity of the method was tested by comparing the chromatograms of six different batches of blank human plasma. There was no chromatographic interference from endogenous compounds at the retention times of AM, IM, and IS. The chromatographic peaks were well resolved to baseline.

Linearity and range

The linearity of the calibration curve for AM and IM in spiked drug-free plasma over the concentration range of 20–2000 ng/mL was evaluated. Ten concentration values (20, 40, 100, 200, 500 and 1000, 1500 and 2000 ng/mL of AM and IM) were spiked in human plasma were analyzed by the HPLC method. Linearity was observed for AM and IM between 20–1600 ng/mL, with correlation coefficient (r2) of 0.998. There was a good correlation between analyte peak area and concentration.

LLOQ

The LLOQ was found to be 0.04 and 0.05µg/mL for AM and IM, calculated on the basis of an S/N ratio of 1:3. The LLOQ was the lowest concentration where RSD and bias were ≤20%.

Precision and accuracy

Results of repeatability, intermediate precision, and accuracy expressed in terms of absolute bias (µg/L) or relative bias (percentage) obtained are summarized inTable1. The within-run and between-run RSDs were <5% for AM and IM. The overall bias was lesser than 5%for both components.

The accuracy of the results obtained by the HPLC method was estimated by measuring the total error. The total error was

determined by 90% expectation tolerance intervals for each concentration level of the validation standards. The upper and lower-expectation tolerance limits in micrograms per liter are presented in Table 1 for each reference concentration level.

Extraction recovery

The mean (±SD) percentage extraction recoveries of AM, IM and IS at the three concentration levels (8, 16, and 32µg/mL for AM and 1, 2, and 4µg/mL for IM) were studied. The use of an IS in the extraction procedure is crucial to compensate for variability in the extraction efficiency. In this study, RT was chosen as the IS because it displayed appropriate chromatographic retention with its peak sufficiently separated from that of AM and IM, and relatively high extraction recovery. The recovery of AM, IM, and IS was consistent and efficient. The mean absolute recovery was 89, 91, and 87 for AM, IM and IS, respectively.

Stability

Post preparation stability and bench-top stability of AM and IM in plasma was investigated at different concentrations (8, 16, and 32 µg/mL for AM and 1, 2, and 4µg/mL for IM). The results revealed that AM and IM in plasma were stable for at least 18 h in the auto sampler and for 5 h at room temperature with average percentage recovery of 89.82 and 93.41%, respectively. After repeated freezing and thawing (three cycles) of plasma samples spiked with AM and IM at three concentration levels (8, 16, and 32 µg/mL for AM and 1, 2, and 4µg/mL for IM), the mean recovery was 89.95 and 90.60%, respectively.

Dilution integrity

The dilution integrity experiment was performed with a higher analyte concentration above the upper limit of quantification, which maybe encountered during real subject sample analysis. The precision and accuracy values for one-fifth and one-tenth dilution ranged from 3.26–3.34 and 98.2–109.7% d, days (runs); n, number of replicates for AM and IM respectively. The percentage of back-calculated concentrations against freshly spiked calibration standards were found in the range of 87.7–101.8% for AM and 89.50– 103.4% for IM.

Results of the Bayesian analysis

The accuracy of analytical results generated by the quantitative analytical method was obtained by applying the Bayesian procedure described in Section 2.3 by defining the acceptance limits within which the results must fall. These acceptance limit was set at a maximum of ±20% around the reference concentration values (T, i) of the validation standards. The minimum accuracy criterion (min) was set at 90%, meaning that the minimum probability of obtaining future measurements falling within the specification limits during routine analysis is 0.90. Having set these requirements, the accuracy profile can be worked out, as illustrated in Fig. 2 A and B for both analytes, respectively. In these profiles, the minimum accuracy criterion (min) of 90% is shown by the dotted horizontal lines. The concentration levels with at least 90% accuracy define the valid concentration range for CF and OF consequently represents the lower and upper quantification limits.

The Bayesian accuracy profile provides an effective way to evaluate the accuracy probability of the HPLC–UV method for the quantification of AM and IM in human plasma. The Figure 2. Bayesian accuracy profiles obtained for analysis of (A) AM and (B) IM. valid concentration range that would produce reliable analytical results for AM and IM along with results of comparison study between samples prepared within Bayesian range and samples prepared out of Bayesian range is given in Table 3.

Results for the comparison study of validation protocols

A comparison between the usual validation protocol and the newer protocol in terms of percentage relative error has been carried out to test the applicability of the new strategy. Significant difference between the two approaches was found. The percentage relative error resulted in usual protocol was more than ±10%, while in the new approach it was within±10%. Accuracy profiles obtained through the two approaches for AM and IM are given in Fig. 3.

CONCLUSION

Analytical method validation plays a vital role in the field of drug analysis, as the generated analytical results are used to make critical decisions pertaining to the conformity of products with release or legal specifications. Hence, perfection in analytical method validation is essential in the present day's analytical scenario. In the proposed research, a HPLC method has been developed and validated using a novel strategy known as Bayesian analysis. In the process, Bayesian analysis determines the concentration range that may produce reliable analytical results by producing better precision values in the measurements. Matrices containing the drugs under study were estimated using the optimized chromatographic conditions. A comparison of results in terms of total error between the newer validation strategy and usual validation protocol has been carried out for further clarification. The results obtained from the study indicated that the newer approach proposed in this research can produce more accurate results and can convert the process of validation most trustworthy.

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Table.1. General validation results for determination of AM and IM human plasma.

Conc. range (ng /ml)	Trueness (d = 3, n = 4)[a] Relative bias (%)	Precision (d = 3,n = 4) Repeatability (RSD%)	Intermediate precision (RSD%)	90% β-expectation tolerance limits
		Amlodipine		
25	4.7	-4.5	-3.9	[24; 27]
50	-3.7	-4.8	-4.7	[56; 59]
100	5.9	3.9	-4.9	[109; 97]
200	2.9	-3.8	3.5	[222; 213]
400	-6.6	-4.7	5.2	[435; 389]
		Indapamide		
75	2.7	2.9	4.3	[81; 79]
150	-3.58	-4.5	-4.3	[160; 162]
300	4.7	-3.9	-3.7	[332;287]
600	-5.8	5.3	-3.5	[665;618]
1200	4.7	-6.1	5.3	[1279;1249`]

[a] d = days (runs) and n = no. of replicates.

Table. 2. Polynomial statistical analysis of the analytical error functions for AM and IM

Variable regression	*Amlodipine*				*Idapamide*			
	r^2	*SE*	*F*	*P*	r^2	*SE*	*F*	*P*
C^2	0.353	0.421	2.352	1.27×10^{-3}	0.268	0.127	1.364	0.38×10^{-4}
C^3	0.367	0.397	2.378	1.26×10^{-5}	0.268	0.321	2.787	1.38×10^{-4}
Coefficient values	A_0	A_1	A_2	A_3	A_0	A_1	A_2	A_3
C^2	0.421	0.479	1.37	2.268×10^{-5}	0.368	0.357	1.232	1.29×10^{-3}
C^3	0.342	0.622	2.267	3.677×10^{-4}	0.257	0.265		-1.5×10^{-3}

Analytical error function: SD (µg/ml) = $A_0 + A_1C + A_2C^2 + A_3C^3$
Amlodipine; SD (µg/ml) = $0.1543 + 0.0328\ C - 3.654 \times 1.06 \times10^{-4}C^2$
Indapamide; SD (µg/ml) = $0.0873 + 0.0876\ C - 2.4\times10^{-3}\ C^3$

Table. 3. Results of analytical error function for Amlodipine and Indapamide

Drugs	**Analytical error function**	
	Linear conc. range considered for procedure (ng/ml)	Conc. range selected for Calibration curve (ng/ml)
Amlodipine	25 – 400	80 – 340
Indapamide	75 –1200	97 –924

*(n=5)

Figure 1-4. Accuracy profile of (A) Amlodipine and (B) Indapamide analyzed by the usual method of analysis showing larger percentage (negative) bias values; (C and D) analyzed within Bayesian calculations showing lower percentage bias values for AM and IM respectively.