CHAPTER 3

ANALYTICAL METHOD DEVELOPMENT

3.1. Introduction

Analysis is an integral component of preformulation and formulation development research. It is essential to have a validated, stability indicating/ specific method of analysis for the drug for which the drug delivery system is to be designed. If a suitable method, for the need, is not available then it is imperative to develop an appropriate, simple and sensitive method for the selected drug to aid during various steps of formulation design. Any newly developed method needs to be validated to prove its appropriateness for the intended purpose. Validation of each assay or test method is performed on a case-by-case basis, to ensure that the parameters are appropriate for the method's intended use. This is even more important when validating stability indicating/ specific assay methods as they may require forced degradation, samples spiked with known degradants, etc [US FDA, 1987a; US FDA, 1987b; ICH, 2005].

Ultra violet (UV)/ visible spectrophotometric technique is one of the earliest and most widely applied detection techniques for drug estimation. It is used extensively, both as stand alone bench top method of analysis, or as a detection technique for methods such as high performance liquid chromatography (HPLC), fast protein liquid chromatography (FPLC), or dedicated blood and plasma analyzers. UV spectrophotometric method is preferred over other techniques for routine analysis as it is less time consuming and also cost effective. Survey of literature revealed only one UV spectrophotometric method and nine chromatographic techniques for estimation of celecoxib. The reported UV spectrophotometric method, for estimation of celecoxib in pure form and in pharmaceutical dosage form, employed 50 % v/v acetonitrile in phosphate buffer (pH 5.6) as the solvent system and 251 nm as wavelength of detection [Saha et al., 2002]. Brief descriptions of the reported chromatographic techniques have been given in the following paragraph in a chronological order.

A reversed phase (RP), isocratic liquid chromatographic (LC) method was developed for determination of celecoxib in bulk drugs and in pharmaceutical dosages using

5-methyl 2-nitro phenol as the internal standard [Srinivasu et al., 2000]. Another method involving normal phase HPLC with column switching and UV detection was developed by Rose and co-workers [Rose et al., 2000]. An LC-mass spectrometric (MS) assay was developed for estimation of celecoxib in human plasma using sulindac as an internal standard [Abdel-Hamid et al., 2001]. An HPLC method using UV detection for determination of celecoxib in serum was developed using tolbutamide as the internal standard and C-18, Wakosil, column. The mobile phase comprised of 10 mM potassium dihydrogen ortho phosphate (pH 3.2) and acetonitrile (50:50 v/v) and a flow rate of 1 ml/min was employed [Jayasagar et al., 2002]. Another HPLC method using the demethylated analogue as internal standard and fluorescence detection was reported. Separation was achieved on a Prontosil C-18 AQ column (150 × 3 mm I.D., 3 µm particle size) at a flow-rate of 0.35 ml/min using water-acetonitrile (40:60, v/v) as the mobile phase [Schonberger et al., 2002]. A micellar electrokinetic chromatographic (MEKC) method was developed for the quantification of celecoxib in pharmaceutical dosage forms using a 25 mM borate buffer (pH 9.3), 25 mM sodium dodecyl sulphate with an extended light path capillary (48.5 cm × 50 µm I.D., 40 cm to detector) and 2-nitro aniline as the internal standard [Srinivasu et al., 2002]. An RP-HPLC using RP-C8 column and 65:35 mixture of acetonitrile: water as the mobile phase with UV detection was developed and validated for estimation of celecoxib in pharmaceutical dosage forms [Saha et al., 2002]. Another method based on RP-HPLC coupled to atmospheric pressure chemical ionization (APCI) mass spectrometry after liquid-liquid extraction, using rofecoxib as internal standard, was developed [Werner et al., 2002]. In addition, an isocratic RP-HPLC method was developed for separation and simultaneous determination of COX-2 inhibitors, viz., celecoxib, rofecoxib, valdecoxib, nimesulide and nabumetone, using 4-chloro-2-nitroaniline as internal standard. In this method good chromatographic separation was achieved using an RP Inertsil C-18 column with mobile phase consisting of methanol and 0.05 % aqueous glacial acetic acid (68:32 v/v) using photodiode array detector [Rao et al., 2005].

Based on literature survey it was concluded that UV spectrophotometric method developed by Saha and his coworkers appeared suitable for the determination of celecoxib in pure form and in pharmaceutical dosage form [Saha et al., 2002]. However, the method gave false positive results when used for analysis of drug in dissolution media containing surfactant (sodium lauryl sulphate, SLS) as employed in the present study for in vitro release study.

Literature survey did not reveal any reported UV spectrophotometric method for estimation of acyclovir. Only three chromatographic techniques for estimation of acyclovir have been reported. An RPLC method, using hexylamine as silanol masking agent, was applied for quantifying acyclovir in liposomal formulations. The method employed a Spherisorb ODS (250 × 4.6 mm I.D.) analytical column, 95 % aqueous phosphate buffer (pH 3.0) and 5 % HPLC grade methanol as the mobile phase and employed UV detection at 254 nm [Caamano et al., 1999]. A sensitive plasma assay for acyclovir, using high performance capillary electrophoresis with sample stacking, which was a viable alternative to HPLC because of its high separation and sensitivity, reproducibility, and adaptability to other nucleoside analogs, was developed and validated by Vo and his group [Vo et al., 2002]. To carry out bioavailability and bioequivalence studies, an HPLC method, employing 18 % acetonitrile, sodium dodecylsulphate 5 mM and phosphate buffer at pH 2.6 as the mobile phase, LiChrospher 100 RP-18 column and UV detection at a wavelength range of 250-260 nm was developed and validated [Fernandez et al., 2003].

Most of these reported methods require extraction of analyte from the respective sample matrices and/ or complicated sample preparation steps and/ or use of internal standards for analysis, thus increasing the time required and error in recovery. None of the reported methods, on detailed study, was found to be suitable for estimation of celecoxib or acyclovir for routine analysis like, drug content estimation, release, stability or similar studies for the intended research work. A UV spectrophotometric method would offer greater advantage in such situations as being simple and rapid for routine analysis. The only reported UV spectrophotometric method for estimation of celecoxib [Saha et al., 2002] failed to solve the intended purpose due to interference from SLS in estimation of the drug. It was considered necessary to develop suitable and sensitive methods for the estimation of both the drugs respectively for pure drug analysis, assay of formulations, and analysis of in vitro release samples.

Developed methods had to be sensitive, specific, stability indicating/ specific, cost effective and quick with minimal sample preparation steps. Method should be free from interference from commonly employed excipients and at the same time, solvent system should be easy to prepare. Methods developed had to be suitable for and adaptable to specific applications related to the present thesis work.

In this chapter, results of UV spectrophotometric analytical method development and validation for estimation of celecoxib and acyclovir are presented. Two simple, sensitive, accurate precise and rapid UV-visible analytical methods having linearity range of

2-20 µg/ml and low limits of detection (DL) and quantitation (QL), were developed for estimation of celecoxib in 0.1 N HCl with 1.0 % w/v SLS at λ_{det} (wavelength of detection) of 255 nm and acyclovir in 0.1 N HCl at λ_{det} of 257 nm respectively. The developed methods were used to estimate the total drug content in two commercially available oral formulations of celecoxib and acyclovir respectively and recovery studies were carried out. The methods were validated by statistical methods as per ICH guidelines-2005 and USP-2000. The developed methods were later used to estimate the drug in pure form, designed formulations, stability samples and samples of in vitro release studies. Forced degradation studies of the analyte were carried out to ensure that the developed methods are stability specific.

3.2. Experimental section

3.2.1. Materials

Celecoxib and acyclovir were obtained as gift samples from IPCA Labs, Mumbai and Medicorp Tech (I) Ltd., Hyderabad respectively. Spectroscopic grade hydrochloric acid (HCl) and acetonitrile (ACN) were purchased from Merck, Mumbai and analytical grade sodium lauryl sulphate (SLS) was purchased from Qualigens, Mumbai. Triple distilled water (TDW) was prepared using our in-house glass distillation unit (fabricated at National Physical Laboratory, New Delhi). Two commercial formulations each of celecoxib (CELACT capsule, Sun Pharmaceuticals Ltd., Baroda and COLCIBRA capsule, Croslands, Mumbai) and acyclovir (CYCLOVIR tablet, Cadila Healthcare, Ahmedabad and ZOVIRAX tablet, Glaxo Smithkline, Nasik) were selected from the local market on random basis. CELACT and COLCIBRA capsules contained celecoxib 200mg each along with other additives, while CYCLOVIR and ZOVIRAX tablets contained acyclovir 200mg in conjunction with other excipients.

3.2.2. Equipments

UV-visible-NIR (near-infrared) spectrophotometer (*Jasco*, Tokyo, Japan, model V-570), with an automatic wavelength accuracy of 0.1 nm, 10 mm matched quartz cells and built in *Jasco* spectra manager software was used for method development and validation. To establish ruggedness of the methods, UV-visible spectrophotometer (Perkin Elmer, Lambda EZ210) with an automatic wavelength accuracy of 0.1 nm, 10 mm matched quartz cells and built-in Perkin spectra manager software was employed.

3.2.3. Preparation of standard curve

Celecoxib: A 100 µg/ml stock solution of celecoxib was prepared in 0.1 N HCl with 1.0 % w/v SLS by first dissolving 10 mg of the drug in 10 ml of ACN and then, making up the final volume to 100 ml with 0.1 N HCl containing 1.0 % w/v SLS. The λ_{det} of celecoxib was finalized by scanning suitable dilutions of the stock in same solvent system. From the stock solution, various standard dilutions were prepared to obtain solutions of 2, 5, 10, 15 and 20 µg/ml, and their respective absorbance values were measured at fixed λ_{det}. Average absorbance values were determined and standard deviation and % coefficient of variation at each concentration were calculated and the results are listed in Table 3.1. One-way ANOVA test for linearity was carried out by picking six sets of calibration curve data on random basis and the results are presented in Table 3.2 [Duncan et al., 1983; Bolton, 1997]. Results of regression analysis of the calibration data are presented in Table 3.3 of the proposed method for estimation of celecoxib. The stability of celecoxib solutions during analysis was also investigated by analyzing samples at different time intervals up to 48 h by storing them at controlled (25±2 °C; 60±5 % RH), ambient (bench-top) and accelerated (40±2 °C; 75±5 % RH) conditions.

Acyclovir: A 100 µg/ml stock solution of acyclovir was prepared in 0.1 N HCl by first dissolving 10 mg of the drug in 10 ml 0.1 N HCl and then, making up the final volume to 100 ml with the same solvent system in 100 ml volumetric flask. The λ_{det} of acyclovir was finalized by scanning suitable dilutions of the stock in 0.1 N HCl. From the stock solution, various standard dilutions were made to obtain solutions of 2, 5, 10, 15 and 20 µg/ml, and their respective absorbance values were measured at fixed λ_{det}. Average absorbance values were determined and standard deviation and % coefficient of variation at each concentration were calculated and the results are listed in Table 3.4. One-way ANOVA test for linearity was carried out by picking six sets of calibration curve data on random basis and the results are presented in Table 3.5 [Duncan et al., 1983; Bolton, 1997]. Results of regression analysis are presented in Table 3.6. The stability of acyclovir solutions during analysis was also investigated by analyzing samples at different time intervals as done for celecoxib.

3.2.4. Method validation

Following procedures were employed to determine various validation parameters of the developed UV spectrophotometric methods. The results are presented in Tables 3.7 and 3.8 for celecoxib and acyclovir respectively [USP, 2000; ICH, 2005; Singh and Chandran, 2007].

Accuracy: It was assessed using nine determinations over three concentration levels covering the specified range. For this, a 10 μg/ml solution was prepared from stock and analyzed. The remaining solution was divided into 9 sub-parts (10 ml each). To each of these sub-parts, 0.2, 1.0, or 2.0 ml (i.e. 3 concentrations; each in triplicate) of 100 μg/ml stock solutions of respective drug was spiked. The percent recovery was reported by the assay of known added amount of analyte in the sample in each case.

Precision: Repeatability or intra-assay precision and intermediate precision were calculated for series of measurements and expressed as percentage relative standard deviation (% RSD) or coefficient of variation (% CV) for each type of precision investigated.

- **Repeatability/ intra-assay precision**, which expresses the precision under the same operating conditions over a short interval of time, was established by nine determinations covering the entire range of the calibration curve (i.e. 3 concentrations in triplicates).
- **Intermediate precision**, expresses the effects of random events i.e. within-laboratories variations (different days, different analysts, etc.) on the precision of the analytical procedure. It was also determined in each case by analyzing nine determinations covering entire range of calibration curve in triplicate for each cause of randomness.

Specificity: Series of twelve samples of the drug at 10 μg/ml concentration was prepared from the stock solution in respective solvent system and analyzed. To evaluate if the developed methods are stability specific, the pure drugs were degraded completely by heating powdered drug at 90 ºC for up to 4 hours. A 10 μg/ml dilution of the degraded sample in the solvent system chosen was made and scanned.

Linearity: Linearity was established by preparing and analyzing five separate series of solutions of the respective drug having concentrations ranging from 2-20 μg/ml and making a plot of absorbance against drug concentration. Least square regression-analysis was done to establish correlation coefficient, y-intercept, slope of the regression line, residual sum of squares and other regression parameters [Tables 3.3 and 3.6]. Linearity range was established by confirming that the method provides an acceptable degree of linearity, accuracy and precision when applied to samples containing concentrations of analyte within or at the extremes of the specified range (2-20 μg/ml) of the analytical procedure.

Detection limit (DL) and **Quantitation limit (QL):** DL and QL were determined on the basis of standard deviation of the response, based on nine separate determinations of absorbance values of the blank, and the slope of the regression equation based on replicate

determinations. They were calculated using the following relationships: DL = 3.3 s/S and QL = 10 s/S respectively, where s = standard deviation of the blank response and S = slope of the calibration curve. In addition, experiments were performed to analyze known concentrations of analyte and establish the minimum level at which the analyte can be quantified with acceptable accuracy and precision by the proposed methods.

Robustness: Robustness was determined by varying the source of water employed for preparing the dissolution media.

Ruggedness: Ruggedness was determined for the developed methods by varying the analyst and the instrument.

Sample solution stability: Stability studies of the drugs, in their respective solvent systems, were carried out at controlled (25±2 °C; 60±5 % RH), ambient (bench-top) and accelerated (40±2 °C; 75±5 % RH) conditions for up to 48 hours [Table 3.9 and 3.10].

3.2.5. Effect of potential formulation excipients on the UV absorbency of the drugs

Effects of potential formulation excipients were seen on the UV absorbency of each of the drugs. Drug and excipients were blended in appropriate ratios (1:1, 1:0.5 or 1:0.1). Aliquot quantity of drug alone and the prepared admixtures were weighed and dissolved in volumetric flasks to prepare 100 µg/ml drug stock solutions. These solutions were filtered using Whatman filter paper no. 1, diluted ten times and analyzed to check whether the presence of those excipients increase/ decrease the absorbance values at λ_{det}. In case of liquid excipients, aliquot volume was pipetted out into the 10 ml volumetric flask along with 1 ml of stock solution of the drug before making up the volume to 10 ml. Excipients selected included: ethyl alcohol, isopropyl alcohol, talc, citric acid, sodium bicarbonate, calcium carbonate, sodium alginate, guar gum, xanthan gum, gelatin, hydroxy propyl methyl cellulose (HPMC) 15, 4000, 15000 and 1 lac cps, ethyl cellulose (EC) 10 cps, carboxy methyl cellulose (CMC), carbopol 934P NF, polycarbophil (Noveon AA1), polyvinyl pyrrolidone (PVP) K-30 and K-90 and ethyl vinyl acetate (EVA).

3.2.6. Estimation of celecoxib and acyclovir respectively from two commercial preparations by the proposed methods

Celecoxib: Contents of twenty capsules of CELACT and COLCIBRA each were crushed, mixed and an aliquot quantity (equivalent to 5 mg of celecoxib) was transferred to a series of 50 ml volumetric flasks (five in each case). To each flask, 5 ml of spectroscopic grade ACN was added, contents were shaken for half an hour on mechanical shaker, and sufficient quantity of 0.1 N HCl containing 1.0 % w/v of SLS was added to fill about 80 % of the volume of the volumetric flasks. The contents in the flask were shaken for 3.5 h more;

volume was made up to 50 ml with same solvent and the contents were filtered through Whatman filter paper no. 1 after thorough shaking. The resulting filtrate was suitably diluted to get final concentration within the limits of linearity for the proposed method. The drug content per capsule of different brands of celecoxib was calculated on average weight basis from the absorbance values obtained (taking average of the five series). The result is tabulated in Table 3.11.

Acyclovir: Contents of two commercial formulations of acyclovir (ACIVIR and ZOVIRAX tablets) were estimated by the proposed method. For each brand of preparation, contents of twenty tablets were crushed, mixed and an aliquot quantity (equivalent to 5 mg of acyclovir) was transferred to a series of 50 ml volumetric flasks (five in each case) and final volume was made using 0.1 N HCl in case of acyclovir. The resulting solutions were thoroughly mixed, filtered through Whatman filter and suitably diluted to get final concentration within the limits of linearity for the proposed method. The drug content per tablet of different brands of acyclovir was calculated on average weight basis from the absorbance values obtained (taking average of the five series). The result is tabulated in Table 3.12.

3.2.7. Recovery Studies

To keep an additional check on the accuracy of the developed assay methods and to study the interference of formulation additives, analytical recovery experiments were performed by adding known amount of pure drug to pre-analyzed samples of commercial dosage forms. The percent analytical recovery values were calculated by comparing concentration obtained for the spiked samples with actual added concentrations. The results are listed in Tables 3.11 and 3.12 for celecoxib and acyclovir respectively.

3.3. Results and discussion

3.3.1. Method development

To develop accurate, precise, sensitive and reproducible UV spectrophotometric methods for celecoxib and acyclovir respectively, various solvent systems such as, water, methanol, acetonitrile, 0.1 N HCl, etc., were tried alone or in combinations or in the presence of surfactants at different proportions. The final decision of using 0.1 N HCl with 1.0 % SLS for celecoxib and 0.1 N HCl for acyclovir respectively, was based on sensitivity, minimal interference, ease of preparation (preference for the same solvent system employed as the dissolution media), suitability for drug content estimation, stability, analysis time and cost. Also the solvent systems employed for analysis of the respective drugs did not show any microbial growth or stability problems during the period of studies. In case of celecoxib,

1.0 % w/v SLS was incorporated to enhance solubility and also because it was used in dissolution media. SLS at 1.0 % w/v concentration did not interfere in analysis and absorbency.

3.3.2. Calibration curve

Celecoxib: The λ_{det} for celecoxib in 0.1 N HCl with 1.0 % w/v SLS was finalized at 255nm. The corresponding UV spectra of celecoxib in 0.1 N HCl with 1.0 % w/v SLS is shown in Figure 3.1. At other wavelengths, either there was interference from the solvent used or correlation coefficient value of the calibration curve data was not good. At 255 nm, the standard drug solution showed a linear relationship with respect to absorbance values obtained in the concentration range of 2-20 μg/ml. The statistical analysis of data obtained revealed high level of precision as can be observed from the low value of standard deviation and low percent of coefficient of variation (% CV) values [Table 3.1].

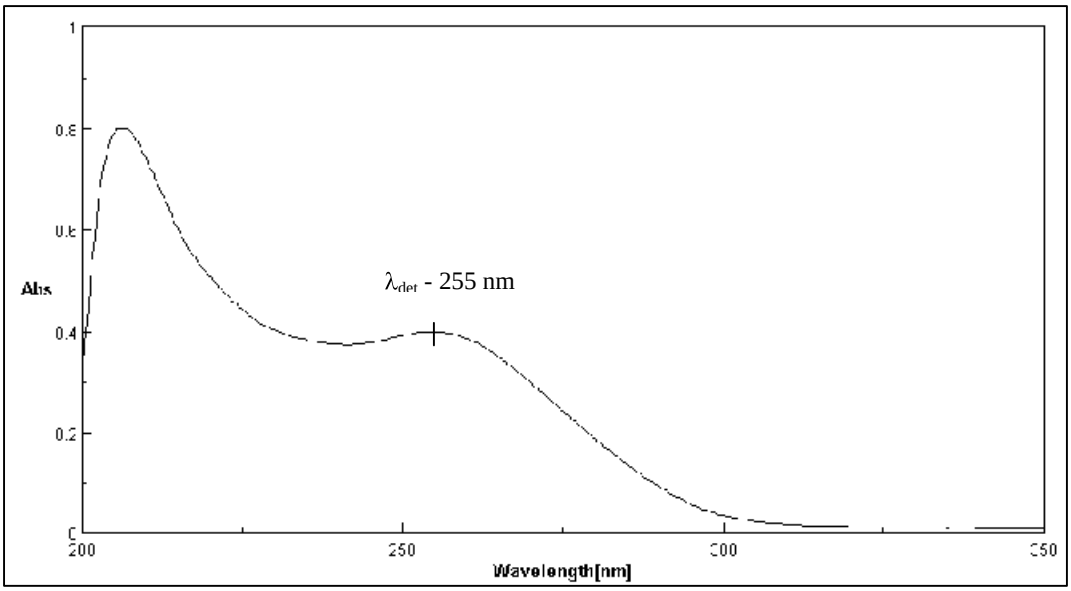

Figure 3.1: Representative UV spectrum of 10 μg/ml solution of celecoxib in 0.1 N HCl with 1 % w/v SLS

Table 3.1: Calibration curve data of the proposed method for estimation of celecoxib in 0.1 N HCl with 1 %w/v SLS at λ_{det} of 255 nm

Concentration (μg/ml)	Mean absorbance ± SD[a]	Coefficient of variation (%)
2	0.0918±0.0011	1.20
5	0.2517±0.0041	1.63
10	0.5400±0.0091	1.69
15	0.8152±0.0153	1.88

20	1.1109 ±0.0246	2.21

[a]: Average of 12 determinations at each concentration with standard deviation

A one-way ANOVA test for linearity was performed, based on the values observed for each pure drug concentration, ranging from 2-20 μg/ml during 6 replicate measurements of the standard solutions. The calculated F-value (F_{Calc}), where F is the ratio of mean sum of squares between and within groups, was less than the critical F-value (F_{Crit}) at 5 % level of significance [Table 3.2].

Table 3.2: Results of one-way ANOVA test for linearity for calibration curve data of celecoxib

Source of variation	**Sum of squares**	**Degree of freedom**	**Mean sum of squares**	**F-calc**	**F-crit**[a]
Between groups	0.004573	5	0.000915	0.0056	2.6207
Within groups	3.951221	24	0.164634		
Total	3.955794	29			

[a]: Theoretical value of F(5, 24) at p= 0.05 level of significance

The linear regression equation obtained was: Abs. = 0.0566 × Conc. - 0.0268, where Abs. is the absorbance and Conc. is the concentration (in μg/ml) of celecoxib [Table 3.3]. Linearity of the regression equation and negligible scatter of points were demonstrated from high correlation coefficient and low residual sum of squares respectively. The obtained slope value without intercept fell within the 95 % confidence limits for slope suggesting that the calibration line of celecoxib in 0.1 N HCl with 1.0 % w/v SLS did not deviate from the origin. The precision of the fit was further confirmed from the low standard error values of the intercept, slope and the estimate [Table 3.3].

Table 3.3: Results of least square regression analysis of UV method for estimation of celecoxib

Statistical Parameters	**Results**
Regression equation[a]	Abs. = 0.0566 × Conc. - 0.0268
Correlation coefficient (r)	0.9999
Residual sum of squares	1.32×10^{-4}
Standard error of slope	4.53×10^{-4}
Standard error of intercept on ordinate	5.57×10^{-3}
Standard error of estimate	1.69×10^{-2}
95 % confidence interval of slope	5.52×10^{-2}, 5.80×10^{-2}
95 % confidence interval of intercept	-4.45×10^{-2}, -9.06×10^{-3}

Slope without intercept	5.48×10^{-2}

[a]: Based on 12 sets of calibration values where, Abs.= Absorbance; and Conc.= Concentration of the drug in μg/ml

Acyclovir: The λ_{det} for acyclovir in 0.1 N HCl was finalized at 257 nm. The corresponding UV spectra in 0.1 N HCl is shown in Figure 3.2.

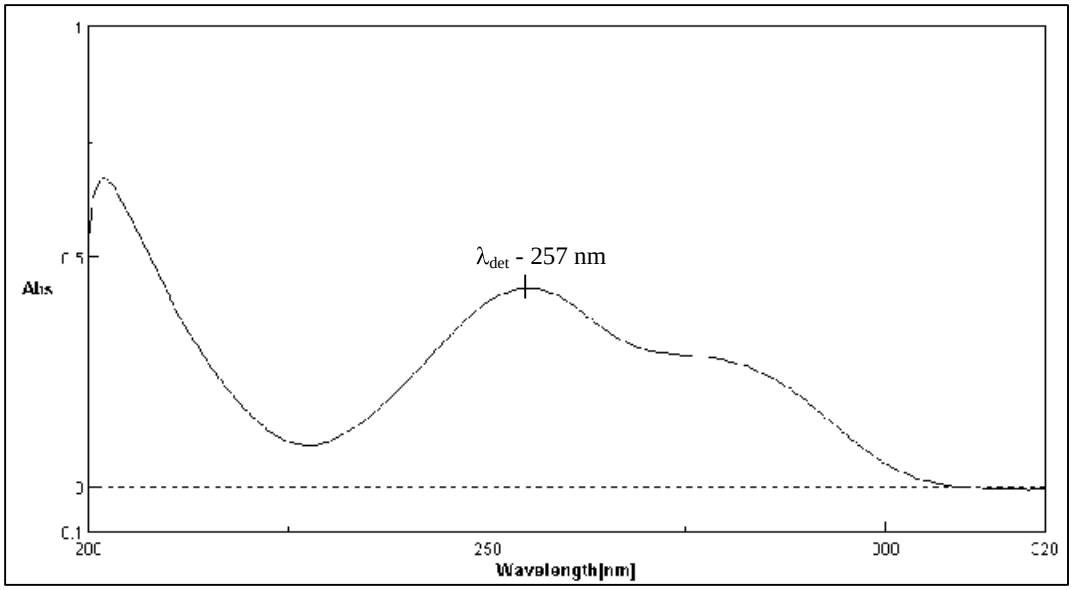

Figure 3.2: Representative UV spectrum of 10 μg/ml solution of acyclovir in 0.1 N HCl

At other wavelengths, either there was interference from the solvent used or correlation coefficient value of the calibration curve data was not high. At 257 nm, drug concentration showed a linear relationship with respect to absorbance values obtained in the concentration range of 2-20 μg/ml [Table 3.4]. The statistical analysis, as mentioned in celecoxib, of data obtained revealed high level of precision as can be observed by the low value of standard deviation and coefficient of variation [Table 3.4].

Table 3.4: Calibration curve data of the proposed method for estimation of acyclovir in 0.1 N HCl at λ_{det} of 257 nm

Concentration (μg/ml)	Mean absorbance ± SD[a]	Coefficient of variation (%)
2	0.1116±0.0018	1.61
5	0.2812± 0.0037	1.32
10	0.5576±0.0158	2.83
15	0.8287±0.0189	2.28

20	1.1068 ±0.0267	2.41

[a]: Average of 12 determinations at each concentration with standard deviation

A one-way ANOVA test for linearity was performed, based on the values observed for each pure drug concentration, ranging from 2-20 μg/ml during 6 replicate measurements (chosen at random) of the standard solutions. The calculated F-value (F_{Calc}) was less than the critical F-value (F_{Crit}) at 5 % level of significance [Table 3.5]. The linear regression equation obtained for acyclovir analysis was: Abs. = 0.0552 × Conc. + 0.0036, where Abs. is the absorbance and Conc. is the concentration (in μg/ml) of acyclovir [Table 3.6]. Linearity of the regression equation and negligible scatter of points were demonstrated from high correlation coefficient and low residual sum of squares respectively. The obtained slope value without intercept fell within the 95 % confidence limits suggesting that the calibration line of acyclovir solution in 0.1 N HCl did not deviate from the origin. The precision of the fit was further confirmed from the low standard error values of the intercept, slope and the estimate [Table 3.6].

Table 3.5: Results of one-way ANOVA test for linearity for calibration curve data of acyclovir

Source of variation	**Sum of squares**	**Degree of freedom**	**Mean sum of squares**	**F-calc**	**F-crit[a]**
Between groups	0.007798	5	0.001560	0.0091	2.6207
Within groups	4.132804	24	0.172200		
Total	4.140602	29			

[a]: Theoretical value of F(5, 24) at p= 0.05 level of significance

Table 3.6: Results of least square regression analysis of UV method for estimation of acyclovir

Statistical Parameters	**Results**
Regression equation[a]	Abs. = 0.0552 × Conc. + 0.0036
Correlation coefficient (r)	0.9999
Residual sum of squares	1.96×10^{-5}
Standard error of slope	1.75×10^{-4}
Standard error of intercept on ordinate	2.15×10^{-3}
Standard error of estimate	2.56×10^{-3}

95 % confidence interval of slope	5.46×10^{-2}, 5.57×10^{-2}
95 % confidence interval of intercept	-3.24×10^{-3}, 1.04×10^{-2}
Slope without intercept	5.54×10^{-2}

[a]: Based on 12 sets of calibration values where, Abs.= Absorbance; and Conc.= Concentration of the drug in µg/ml

3.3.3. Validation of the developed method

The developed methods were validated according to the standard procedures [USP, 2000; ICH, 2005] and the results obtained are tabulated in Tables 3.7 and 3.8 for celecoxib and acyclovir respectively.

Celecoxib: The developed method was found to be accurate, precise and specific. The method showed a linearity range at 2-20 µg/ml at 255 nm in 0.1 N HCl with 1.0 % w/v SLS. Low DL of 0.14 µg/ml and QL of 0.43 µg/ml were obtained. But the actual QL determined on experimental basis was found to be 2.0 µg/ml [Table 3.7]. The % RSD for intra-assay precision (1.32 %) and intermediate precision (2.47 %) was less than 3.0 %, which fall well below the acceptance criteria described [Shah et al, 1991; Singh and Chandran, 2007]. Changing the source of water, from triple distilled water to demineralized potable water, for preparation of the dissolution media increased the intercept and slightly decreased the slope. But the method was quite rugged as varying the analyst as well as varying the instrument had insignificant impact on the accuracy. Ruggedness was found to be 99.81±2.47 % [Table 3.7]. After degrading pure drug completely by heating it at 90 ºC for 4 h and scanning appropriate dilution of the degraded product, the scan obtained was almost flat, touching the ?-axis with no absorbance at λ_{det} of celecoxib. Thus, the method was found to be stability specific [Figure 3.3].

Table 3.7: Analytical validation parameters of UV method for estimation of celecoxib

Analytical parameters		**Results**
Accuracy (%)		99.81±1.32
Precision (% RSD)	Repeatability/ Intra-assay precision	1.32
	Intermediate precision	2.47
Specificity		A 10 ug/ml solution in 0.1 N HCl with 1% w/v SLS at 255 nm will show an absorbance of 0.5400±0.0091
Stability specific		Yes
Linearity range (µg/ml)		2-20
Detection limit (µg/ml)	Based on the standard deviation of the response and the slope	0.14
Quantitation limit	Based on actual analysis	2.00

(µg/ml)	Based on the standard deviation of the response and the slope	0.43
Robustness		Not robust
Ruggedness (%)		99.81±2.47
Sample solution stability		Stable up to 48 h under bench top condition

RSD- Relative standard deviation

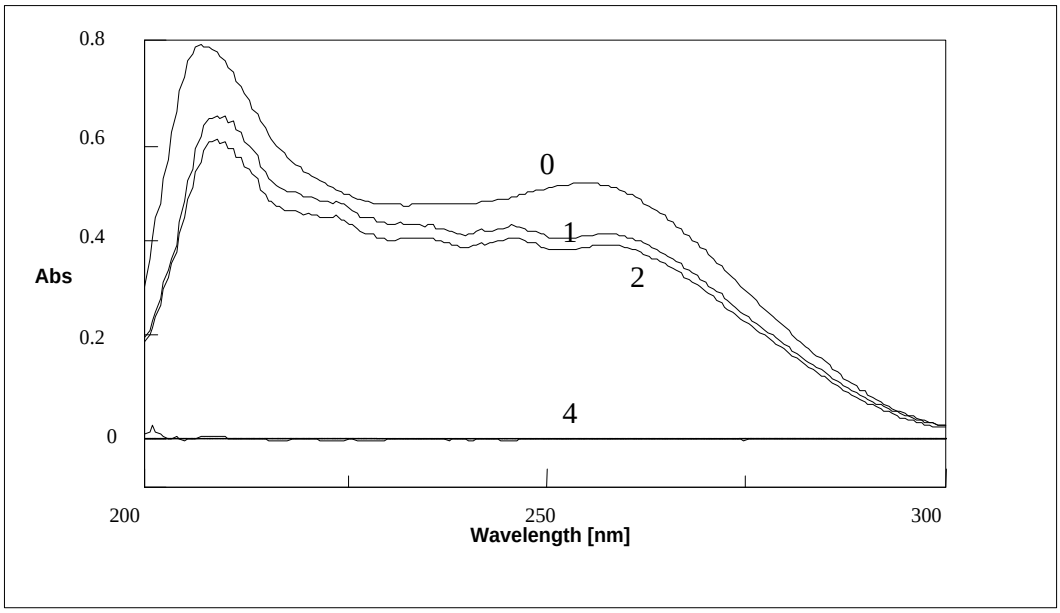

Figure 3.3: Overlay UV spectrum of 10 µg/ml solution of celecoxib after heating dry powder at 90 ºC for 0, 1, 2 and 4 h

Acyclovir: The proposed UV method for analysis of acyclovir was also found to be accurate, precise and specific. The method had a linearity range of 2-20 µg/ml at 257 nm in 0.1 N HCl. Low DL of 0.17 µg/ml and QL of 0.51 µg/ml were obtained. The actual QL determined on experimental basis was found to be 2.0 µg/ml [Table 3.8]. The % RSD for intra-assay precision (1.63 %) and intermediate precision (2.81 %) was below the acceptance criteria of 3.0 % reported earlier. Changing the source of water, from triple distilled water to demineralized potable water, for preparation of the dissolution media increased the intercept and slightly decreased the slope, as in the previous method. But this method was also quite rugged as varying the analyst as well as the instrument had insignificant impact on the accuracy. Ruggedness was found to be 101.50±2.81 % [Table 3.8]. After degrading pure drug completely by heating it at 90 ºC for 3.75 h and

scanning appropriate dilution of the degraded product, the scans obtained were almost flat, touching the ?-axis with no absorbance at λ_{det} of acyclovir. Thus, the method was found to be stability specific [Figure 3.4].

Table 3.8: Analytical validation parameters of UV method for estimation of acyclovir

Analytical parameters		**Results**
Accuracy (%)		101.50±1.65
Precision (% RSD)	Repeatability/ Intra-assay precision	1.63
	Intermediate precision	2.81
Specificity		A 10 ug/ml solution in 0.1 N HCl at 257 nm will show an absorbance of 0.5576±0.0158
Stability specific		Yes
Linearity range (µg/ml)		2-20
Detection Limit (µg/ml)	Based on the standard deviation of the response and the slope	0.17
Quantitation Limit (µg/ml)	Based on actual analysis	2.00
	Based on the standard deviation of the response and the slope	0.51
Robustness		Not robust
Ruggedness (%)		101.50±2.81
Sample solution stability		Stable up to 48 h under bench top condition

RSD- Relative standard deviation

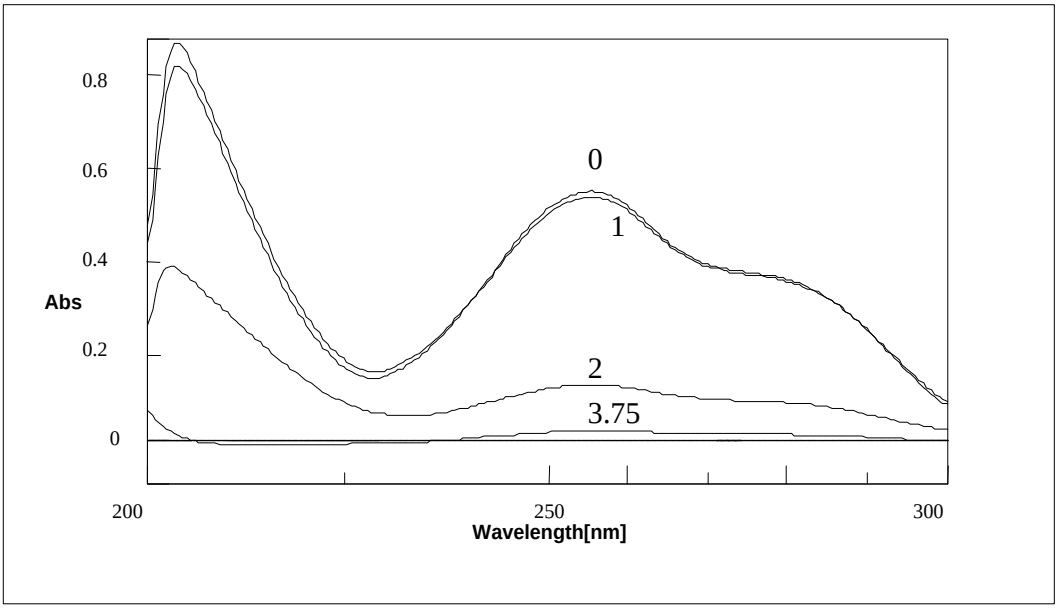

Figure 3.4: Overlay UV spectrum of 10 μg/ml solutions of acyclovir after heating dry powder at 90 ºC for 0, 1, 2 and 3.75 h

3.3.4. Sample solution stability studies

Overlay scans obtained at zero time, 12, 24 and 48 h revealed no degradation up to 48 h, in the selected solvent system of 0.1 N HCl with 1% w/v SLS for celecoxib and 0.1 N HCl for acyclovir, at controlled, ambient and accelerated conditions for either of the drugs [Tables 3.9 and 3.10]. The observed stability of the drugs in selected media up to 48 h will be useful in carrying out long-term (24 h) dissolution/ release studies.

Table 3.9: Results of stability studies of celecoxib at controlled, ambient and accelerated conditions

Media	**Condition**		
	Controlled	**Ambient**	**Accelerated**
0.1 N HCl with 1 % v/v Tween 80	Slight depreciation in absorbency profile after 24 h	Slight depreciation in absorbency profile after 24 h	Slight depreciation in absorbency profile after 24 h
0.1 N HCl with 1% w/v SLS	No difference in the overlayed spectra up to 48 h	No difference in the overlayed spectra up to 48 h	No difference in the overlayed spectra up to 48 h

Table 3.10: Results of stability studies of acyclovir at controlled, ambient and accelerated conditions

Media	Condition		
	Controlled	Ambient	Accelerated
0.1 N HCl	No difference in the overlayed spectra up to 48 h	No difference in the overlayed spectra up to 48 h	No difference in the overlayed spectra up to 48 h

3.3.5. Effect of potential formulation excipients on the UV absorbency of the drugs

None of the selected excipients/ reagents/ chemicals, except for EVA, had an appreciable effect on the UV absorbency profile in case of either of the drugs. Absorbance values at λ_{det} of the respective drugs remained the same in the presence of aliquot amount of the excipients studied. Only in case of EVA, there was an increase in the absorbance values at ?$_{det}$ for both the drugs.

3.3.6. Drug content estimation and recovery studies

The developed methods were successfully used for estimation of drug contents in commercial formulations. Drug content was found to vary from 197.62±0.94 to 200.60±1.02 mg/capsule for celecoxib and from 194.2±0.76 to 197.62±0.92 mg/tablet for acyclovir. The estimated drug content showed low standard deviation and % CV, further establishing precision of the proposed methods and also suggesting non-interference from the formulation matrix/ additives present in the studied formulations. The accuracy of the results of estimation was further tested by recovery experiments. The average recovery varied from 99.81±0.23 to 101.42±0.20 % in case of celecoxib and 99.38±0.19 to 101.22±0.21 % in case of acyclovir. The results are presented in Tables 3.11 and 3.12 respectively for celecoxib and acyclovir.

Table 3.11: Results of drug content estimation and analytical recovery for celecoxib

Sample	Label claim	Drug content ± SD[a]	CV (%)	Analytical recovery ± SD[a] (%)
Pure soln[b]	-	99.90±0.16 µg/ml	0.16	100.29±0.11
CELACT	200 mg/cap	197.62±0.94 mg/cap	0.48	101.42±0.20
COLCIBRA	200 mg/cap	200.60±1.02 mg/cap	0.51	99.81±0.23

[a]: Average of nine determinations with standard deviation; [b]: 100 µg/ml solution; CV- Coefficient of variation; cap- Capsule

Table 3.12: Results of drug content estimation and analytical recovery for acyclovir

Sample	Label claim	Drug content ± SD[a]	CV (%)	Analytical recovery ± SD[a] (%)
Pure soln[b]	-	99.50±0.11 µg/ml	0.11	99.38±0.19

CYCLOVIR	200 mg/tab	194.2±0.76 mg/tab	0.39	101.22±0.21
ZOVIRAX	200 mg/tab	197.62±0.92 mg/tab	0.47	100.13±0.29

[a]: Average of nine determinations with standard deviation; [b]: 100 μg/ml solution; CV- Coefficient of variation; tab- Tablet

3.4. Conclusions

The developed UV spectrophotometric methods for estimation of celecoxib and acyclovir were simple and rapid with high accuracy, precision, specificity and low DL & QL. Rapidity of both the methods and capability to quantify very low concentrations (2.0 μg/ml) of respective drugs, made them ideal for a variety of analyses, including pure drug analysis, assay of formulations, in vitro release and stability studies. Unlike earlier reported LC methods, the developed UV methods did not utilize a special extraction step for recovering the drug from the formulation excipients matrices, and thereby decreased the degree of error, time for estimation and the overall cost. Solvent systems employed were such that could be used for in vitro release studies, which had added advantage of decreased sample preparation steps and time consumed. All the excipients studied (except for EVA), in the concentration range used, did not interfere in analysis of the drug. Sample recoveries in all formulation using the above two methods were in good agreement with their respective label claims/ theoretical drug content, thus suggesting the validity of the methods and non-interference of formulation excipients in the estimation. In the selected solvent systems, drugs were stable for more than 48 hours, making it suitable for carrying out long-term (24 h) dissolution/ release studies. The developed methods were found to be stability specific and were found to have acceptable validation parameters as per ICH guidelines-2005, USP-2000 and statistical methods.

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