Original article

Simultaneous estimation of degree of crystallinity in combination drug product of abacavir, lamivudine and neverapine using X-ray powder diffraction technique

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A B S T R A C T

Objective: In the present study, simultaneous determination of degree of crystallinity content in abacavir (ABC), lamivudine (3TC) and neverapine (NVP) from there combination drug product using X-ray powder diffraction (XRPD) technique is developed and validated.
Methods: The X-ray procedure for the identification and determination of the degree of crystallinity in ABC, 3TC and NVP drug product is developed and validated. It is based on the X-ray diffraction from crystalline region of the drug product. The characteristic peaks of the three drugs were characterized using XRPD.
Results: ABC, 3TC and NVP concentrations ranging from 70% to 130% in drug product were prepared and linearity in this concentration range is described. The % coefficient of variation (%CV) was found to be 0.9982 for ABC, 0.9978 for 3TC and 0.9984 for NVP. The mean recoveries were found to be 100.3% for ABC, 99.0% for 3TC and 100.8% for NVP. Regressions statistics and Analysis of variance (ANOVA) table results were evaluated and found to be satisfactory.
Conclusions: The method has been applied to unknown mixtures of drug formulations and stability samples. The proposed method can be useful in the quality control of combination drug products.
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1. Introduction

The managing of human immunodeficiency virus (HIV) includes the use of multiple antiretroviral, since single drug therapy becomes ineffective due to development of HIV resistant strains. According to treatment guidelines antiretroviral regimen should contain at least two nucleoside analogue reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) in a fixed dose combination.[1,2] An example of such combination is ABC, 3TC and NVP, which is very efficient in the treatment of HIV. Abacavir (ABC) is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). ABC is phosphorylated to active metabolites that compete for incorporation into viral DNA. Intracellularly, ABC is converted by cellular enzymes to the active metabolite carbovir triphosphate, an analogue of deoxyguanosine-5′-triphosphate (dGTP). Chemically ABC is [(1S, 4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl] cyclopent-2-en-1-yl] methanol.[3–5] Lamivudine (3TC) is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1) and hepatitis B (HBV). 3TC is a synthetic nucleoside analogue and is phosphorylated intracellularly to its active 5′-triphosphate metabolite, lamivudine triphosphate (L-TP). Chemically 3TC is 4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl]-1, 2-dihydropyrimidin-2-one.[6–8] Neverapine (NVP), is a non-nucleoside reverse transcriptase inhibitor (NNRTI) used to treat HIV-1 infection and AIDS. Neverapine falls in the non-nucleoside reverse transcriptase inhibitor (NNRTI) class of antiretrovirals.[9] Chemically NVP is 11-cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2′,3′-e][1,4]diazepin-6-one.

The objective of the present study is to describe an X-ray diffraction quantitative method for the simultaneous estimation of degree of crystallinity of ABC, 3TC and NVP in a triple composition drug product. The X-ray diffraction pattern consists of information about peak positions and intensity, which are indicative of the crystal structure, composition and symmetry of the contributing phase. The peak intensities reflect the total scattering from the each

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plane in the phase's crystal structure, and are directly dependent on the distribution of particular atoms in the structure. The diffraction intensity equation is summarized below:

$$I_{(hkl)\alpha} = \frac{I_0 \lambda^3}{64\pi r}\left(\frac{e^2}{m_e c^2}\right)^2 \frac{M_{(hkl)}}{V_\alpha^2}\left|F_{(hkl)\alpha}\right|^2$$
$$\left(\frac{1 + \cos^2(2\theta)\cos^2(2\theta_m)}{\sin^2\theta\cos\theta}\right)_{hkl}\frac{\nu_\alpha}{\mu_s}$$

where, $I_{(hkl)\alpha}$ is the Intensity of reflection of hkl in phase α, I_0 is the incident beam intensity, r is the distance from specimen to detector, λ is X-ray wavelength, $(e^2/m_e c^2)^2$ is the square of classical electron radius, μ_s is linear absorption coefficient of the specimen, V_α is volume fraction of phase α, M_{hkl} is multiplicity of reflection hkl of phase α, $\nu\alpha$ is volume of the unit cell of phase α, $2\theta_m$ is the diffraction angle of the monochromator and $F_{(hkl)\alpha}$ is the structure factor for reflection hkl of phase α (i.e., the vector sum of scattering intensities of all atoms contributing to that reflection).[10]

Methods have been developed which use peak intensities for quantitative analysis of diffraction data. Some of the quantitative Methods based on Intensity Ratios of diffraction data are Absorption-Diffraction Method, Method of Standard Additions, Internal Standard Method, Reference Intensity Ratio Methods and Full-Pattern Analysis – the Rietveld Method.[11,12] In the present study regression analysis is carried out to establish the equation that best describes the linear relationship between instrument response (y) and analyte level (x). The relationship is given by the equation of the line, $y = mx + c$ where m is the gradient of the line and c is its intercept with the y-axis. Linear regression establishes the values of m and c, which best describes the relationship between the data sets. The equations for calculating m and c are given as follows:

$$m = \frac{\sum_{i=1}^{n}\{(x_i - \overline{x})(y_i - \overline{y})\}}{\sum_{i=1}^{n}(x_i - \overline{x})^2}$$

$$c = \overline{y} - m\overline{x}$$

The correlation coefficient (R) is a measure of the strength of the degree of correlation between the y and x values. R can take any value between $+1$ and -1; the closer it is to 1, the stronger the correlation. R is given by the following equation.

$$R = \frac{\sum_{i=1}^{n}\{(x_i - \overline{x})(y_i - \overline{y})\}}{\left\{\left[\sum_{i=1}^{n}(x_i - \overline{x})^2\right]\left[\left[\sum_{i=1}^{n}(y_i - \overline{y})^2\right]\right]\right\}^{1/2}}$$

where x_i and y_i are the value on x-axis and y-axis, $\overline{x}$ and $\overline{y}$ are the mean of x_i and y_i values and n is the number of calibration points. The parameters related to R are R^2 and adjusted R^2. R^2 is often used to describe the fraction of the total variance in the data which is contributed by the line that has been fitted. Ideally, if there is a good linear relation, the majority of variability can be accounted for by the fitted line. R^2 value is close to 1. The adjusted R^2 value is interpreted in the same way as R^2 but is always lower. It is useful for assessing the effect of adding additional terms to the equation of the fitted line (e.g., if a quadratic fit is used instead of a linear fit). The R^2 value always increases on the addition of an extra term to the equation, but this does not mean that the extended equation is necessarily a better fit of the data. The adjusted R^2 value is more useful in such cases as it takes account of the reduction in the degrees of freedom, which occurs each time an additional term is added to the equation of the line and therefore does not automatically increase on addition of extra terms. Another important

parameter in regression analysis is Residual standard deviation (or standard error), which is a statistical measure of the deviation of the data from the fitted regression line. It is calculated using the following equation:

$$s(r) = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n-2}}$$

where y_i is the observed value of y for a given value of x_i, $\hat{y}_i$ is the value of y predicted by the equation of the calibration line for a given value of x_i and n is the number of calibration points. Analysis of variance (ANOVA) table for the regression is produced in regression analysis. In ANOVA table the sum of squares terms (SS) represent different sources of variability in the calibration data. The regression term represents the variability in the data that can be accounted for by the fitted regression line. The residual term is the sum of the squared residuals. Each mean square (MS) term is simply the sum of squares term divided by its degrees of freedom. The F value is the ratio of the regression MS term to the residual MS term. The significance F value represents the probability of obtaining the results in the ANOVA table if there is no correlation between y and x values, i.e., obtaining the results by chance. A small value indicates that the results were unlikely to have happened by chance, indicating that it is highly likely that there is a strong relationship between the y and x values. For a calibration curve to be of any use the significance F value should be extremely small. This value is also known as the p-value.[13–17]

To our knowledge, based on extensive review, no efforts have been made in published literature to determine the degree of crystallinity simultaneously in ABC, 3TC and NVP combination drug product, determination of regression statistics and evaluation of ANOVA table parameters.

2. Experimental

The three drugs ABC, 3TC and NVP were conformed using PXRD. The required concentrations ranging between 70% and 130% were prepared accurately using high Sensitive Microbalance (Mettler Toledo XP2U, Max 2.1 g-Min-1°µg).

2.1. PXRD analysis

Diffraction patterns were collected using Bruker D8 advance X-ray diffractometer with Cu anode and Lynx eye detector. ABC, 3TC and NVP were scanned from $3°$ 2θ to $45°$ 2θ, with step size $0.01°$ 2θ and time per step of 0.1 s for the identification purpose. After selecting the non-interfering peak, the patterns of spiked standards were collected from $12°$ 2θ to $21°$ 2θ, with step size $0.01°$ 2θ and time per step of 0.72 s. The instrument was operated at 40 kV generator voltage and 40 mA generator current. Variable divergent slit and Anti scattering slit were used of V_{20} mm, Nickel filter was used in secondary beam path. Eva software was used for data processing and evaluation.

Approved raw material of ABC, 3TC and NVP assigned against primary reference standard is used for the study. The chemical structures are represented in Fig. 1. Spiked standard concentrations were prepared by geometrical mixing of ABC, 3TC, NVP and placebo. Average weight of tablet is 394 mg, which contains about 69 mg of abacavir sulphate which is equivalent to 60 mg of abacavir, 30 mg of lamivudine and 50 mg of neverapine. Eleven standard blend mixtures were prepared whose average weight is calculated about 500 mg for convenient packing in sample holders. The ratio of ABC, 3TC and NVP taken for the method development and quantitative determination of crystallinity in final drug product are tabulated in Table 1.
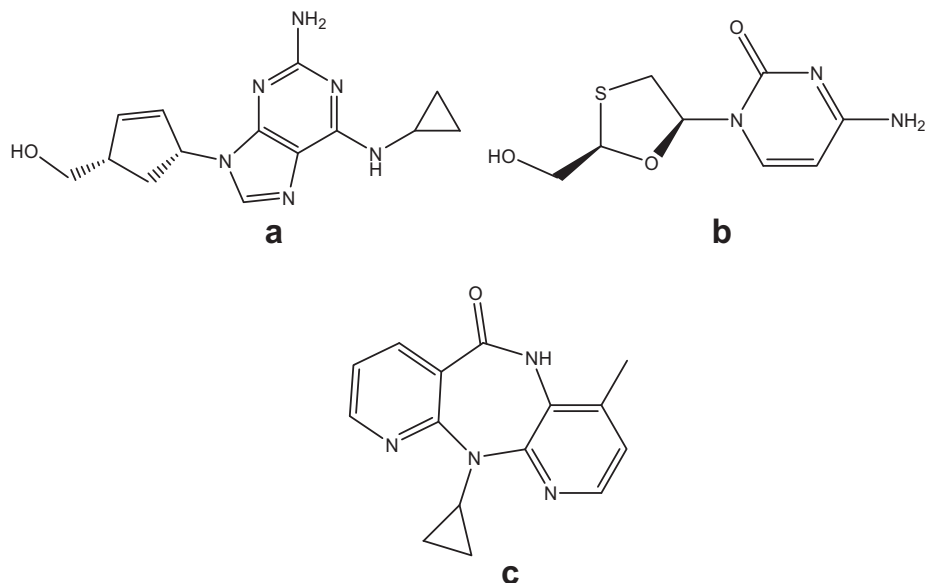
Fig. 1. Chemical structure of Abacavir (a), Lamivudine (b) and Neverapine (c).

% Concentration of ABC, 3TC and NVP in Standard-1 is 69.9, 130.1 and 129.9; Standard-2 is 75.1, 125.3 and 125.2; Standard-3 is 80.1, 120.3 and 120.1; Standard-4 is 84.7, 114.9 and 115.2; Standard-5 is 90.1, 110.2 and 109.7; Standard-6 is 95.3, 105.1 and 105.3; Standard-7 is 100.4, 100.1 and 100.2; Standard-8 is 110.1, 90.1 and 90.3; Standard-9 is 115.1, 85.2 and 85.1; Standard-10 is 120.2, 80.1 and 80.2 and Standard-11 is 129.8, 70.1 and 70.3.

2.2. Method validation

The proposed method was validated with respect to selectivity, linearity, precision and recovery.[18]

2.2.1. Selectivity

The selectivity of the method was determined to make sure that ABC, 3TC and NVP characteristics peaks were well separated and free from interference of Placebo in the drug product.

2.3. Linearity

Standard sample preparations with concentration range of 70%–130% were prepared by spiking ABC, 3TC and NVP in placebo. Standard calibration curve was constructed by plotting the peak areas of ABC, 3TC and NVP against the corresponding

concentrations of standard samples. Linear regression equation and correlation coefficient were determined.

2.4. Precision

The precision of this analytical method is expressed as the relative standard deviation of a series of measurements. Spiked standard concentrations of ABC, 3TC and NVP at 95%, 105% and 110% concentration levels were prepared and scanned for three times.

2.5. Recovery

Three concentrations of ABC, 3TC and NVP in placebo i.e., 95%, 105% and 110% were prepared and three replicates of each concentration are scanned.

3. Results and discussion

The three drugs ABC, 3TC and NVP were identified and characterised using PXRD and the overlay of the diffractogram of the drugs are represented in Fig. 2. The characteristics peaks with their corresponding intensity % of the three drugs are represented in
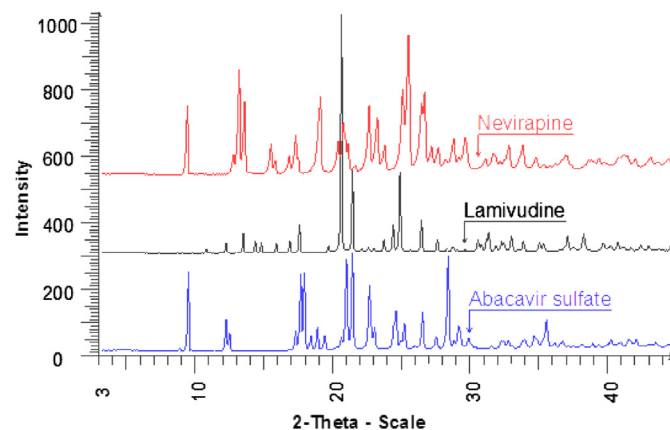
Table 1
Weights of ABC, 3TC, NVP & Placebo taken for the preparation of standard blend mixtures.

Standard blend mixtures	ABC (mg)	3TC (mg)	NVP (mg)	Placebo (mg)	Total weight of the blend (mg)
Standard-1	61.51	49.44	81.84	307.29	500.08
Standard-2	66.09	47.61	78.88	307.52	500.10
Standard-3	70.49	45.71	75.66	308.19	500.06
Standard-4	74.54	43.66	72.58	308.50	499.27
Standard-5	79.29	41.88	69.11	309.90	500.18
Standard-6	83.86	39.94	66.34	310.22	500.36
Standard-7	88.35	38.04	63.13	310.42	499.94
Standard-8	96.89	34.24	56.89	311.85	499.87
Standard-9	101.29	32.38	53.61	311.98	499.26
Standard-10	105.78	30.44	50.53	312.54	499.28
Standard-11	114.22	26.64	44.29	313.94	499.09

Fig. 2. Overlaid diffractograms of ABC, 3TC and NVP.

Table 2
Characteristics peaks of ABC, 3TC and NVP.

Abacavir		Lamivudine		Neverapine	
Angle (2$\theta°$)	Intensity %	Angle (2$\theta°$)	Intensity %	Angle (2$\theta°$)	Intensity %
8.736	5.0	10.666	2.2	9.285	50.3
9.37	81.9	12.143	4.4	10.295	2.7
12.138	34.2	13.396	8.6	12.662	15.6
12.385	19.6	14.279	5.4	13.096	75.6
17.253	21.9	14.731	4.8	13.495	53.2
17.628	80.1	15.836	4.4	15.439	23.3
17.884	80.4	16.816	5.4	15.745	11.7
18.381	17.9	17.529	11.7	16.773	14.8
18.859	26.2	19.669	3.3	17.255	28.9
19.392	17.5	20.612	100.0	17.441	13.8
20.245	6.3	21.426	33.2	18.523	7.5
20.603	15.6	22.124	2.3	18.937	41.4
20.988	94.3	22.597	2.7	19.072	56.6
21.425	100.0	23.005	2.8	20.372	25.1
22.685	68.1	23.746	6.0	20.754	38.0
23.053	25.6	24.418	11.7	20.878	30.5
24.492	30.5	24.922	32.5	21.106	23.7
24.635	42.5	25.683	2.0	21.624	8.1
24.985	13.0	26.491	13.6	22.658	50.4
25.257	29.6	27.669	5.9	23.258	41.4
26.009	7.3	28.39	2.2	23.787	22.4
26.585	40.8	28.771	2.8	25.126	61.3
27.586	16.4	28.875	2.8	25.544	100.0
28.471	98.0	29.096	1.8	26.506	51.7
28.885	12.5	29.697	1.8	26.738	60.0
29.244	27.1	30.656	5.7	27.235	21.4
29.976	15.6	30.885	4.2	27.733	20.7
30.203	8.5	31.297	6.3	28.265	12.3
30.467	6.1	31.442	8.6	28.874	26.9
31.652	6.9	31.964	3.4	29.248	14.2

Table 2. Form this information we can select non-interfering peaks of ABC, 3TC and NVP which are used in the creation of calibration curves.

The standard calibration curves (Fig. 3.) were constructed by plotting spiked standard preparations against Net area (counts per seconds (Cps) × 2$\theta°$). Overlaid diffractograms of different spiked standard preparations are represented in Fig. 4. The standard calibration curve for ABC, 3TC and NVP was linear over a concentration range of 70%–130%. The correlation coefficient (R^2) was 0.9982 for ABC, 0.9978 for 3TC and 0.9984 for NVP, which are well within acceptable limit.[18] Linear regression equations of ABC, 3TC and NVP were found to be $y = 0.0578x - 0.1$, $y = 0.219x - 14.511$ and $y = 0.0832x - 4.9302$ respectively.

Three standard preparations at 95%, 105% and 110% level were prepared and measurements are collected for three replicates and
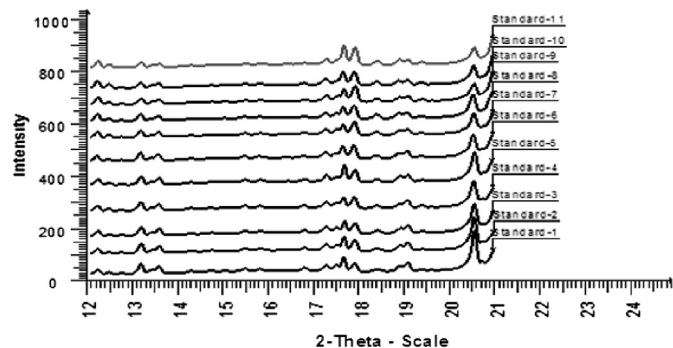
Fig. 4. Overlaid diffractograms of Spiked standard preparations of ABC, 3TC and NVP.

Table 3
Precision, Accuracy and Reproducibility data.

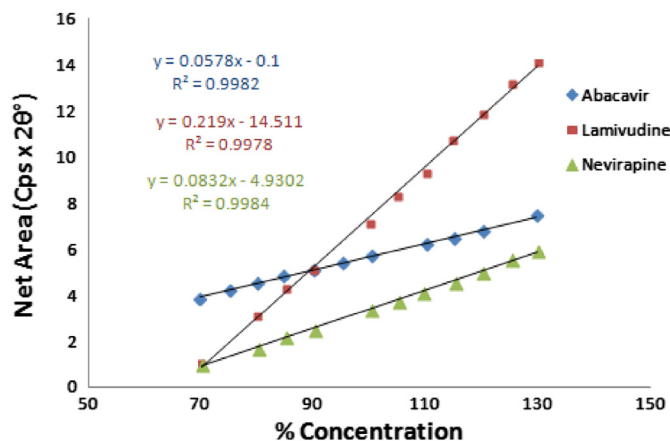| Actual weight %	Calculated weight %					
	ABC	% Recovery	3TC	% Recovery	NVP	% Recovery
95	95.3	100.3	94.5	99.5	94.7	99.7
	95.1	100.1	94.9	99.9	94.9	99.9
	95.4	100.4	94.8	99.8	95.2	100.2
Mean	95.3		94.7		94.9	
%RSD	0.16		0.22		0.27	
105	103.2	98.3	104.9	99.9	106.4	101.3
	106.1	101.0	102.6	97.7	105.7	100.7
	104.7	99.7	103.8	98.9	106.2	101.1
Mean	104.7		103.8		106.1	
%RSD	1.39		1.11		0.34	
110	111.7	101.5	106.9	97.2	112.7	102.5
	113.6	103.3	109.1	99.2	111.9	101.7
	107.5	97.7	108.7	98.8	110.2	100.2
Mean	110.9		108.2		111.6	
%RSD	2.81		1.08		1.14	

corresponding peaks of ABC, 3TC and NVP were integrated for the evaluation of precision, recovery, accuracy and reproducibility (Table 3). The mean recovery values of 100.3% for ABC, 99.0% for 3TC and 100.8% for NVP were obtained. Average recovery for each drug was above 99%. The low %RSD value obtained by the triplicate measurement of each of the concentration of ABC, 3TC and NVP indicates that the method is precise and reproducible.

Regression statistics are evaluated for prepared spiked standard concentrations versus Net area obtained. Parameters like multiple R, R^2, Adjusted R^2 and Standard error are assessed. The correlation coefficient R (Multiple R), R^2 and Adjusted R^2 values for ABC, 3TC and NVP are tabulated in Table 4.

The correlation coefficient (R) for ABC, 3TC and NVP is 0.999083539, 0.998905611and 0.99918471 respectively indicating the stronger relationship. R^2 and adjusted R^2 are the parameters related to R and they provide us the information about fraction of the total variance in data added by the fitted line. Adjusted R^2 value is always lower than R^2 value.

Fig. 3. The standard calibration curves for ABC, 3TC and NVP.

Table 4
Regression statistics of ABC, 3TC and NVP.

Regression Statistics			
	ABC	3TC	NVP
Multiple R	0.999083539	0.998905611	0.99918471
R^2	0.998167918	0.997812419	0.998375602
Adjusted R^2	0.997964354	0.997569355	0.998195114
Standard Error	0.051067604	0.211444399	0.068830267
Observations	11	11	11

Table 5
ANOVA table for the regression of ABV, 3TC and NVP.

	df	SS			MS		
		ABC	3TC	NVP	ABC	3TC	NVP
Regression	1	12.78769363	183.535355	26.20613828	12.78769363	183.535355	26.20613828
Residual	9	0.023471102	0.402378604	0.04263845	0.0026079	0.044708734	0.004737606
Total	10	12.81116473	183.9377336	26.24877673			

F			Significance F		
ABC	3TC	NVP	ABC	3TC	NVP
4903.444352	4105.134271	5531.515396	1.24841E-13	2.77336E-14	7.26413E-15

Coefficients				Standard error		
	ABC	3TC	NVP	ABC	3TC	NVP
Intercept	−0.10002267	−14.511249873	−4.930170812	0.081841343	0.357311531	0.116918398
X variable 1	0.057820554	0.218989614	0.083193883	0.000825718	0.003417904	0.001118586

t-Stat				p-value		
	ABC	3TC	NVP	ABC	3TC	NVP
Intercept	−1.22215334	−40.61231897	−42.16762188	0.252691399	1.656845E-12	1.1832827E-11
X variable 1	70.02459819	64.07132175	74.37415812	1.24841E-13	2.77336E-14	7.26413E-15

Lower 95%				Upper 95%		
	ABC	3TC	NVP	ABC	3TC	NVP
Intercept	−0.28516065	−15.31954471	−5.194658604	0.085115309	−13.70295504	−4.665683021
X variable 1	0.055952651	0.211257779	0.080663466	0.059688457	0.22672145	0.08572430

Table 6
% Crystallinity determination of unknown samples.

% Crystallinity							
	Stability condition	ABC	Net Area (Cps x 2$\theta°$)	3TC	Net Area (Cps x 2$\theta°$)	NVP	Net Area (Cps x 2$\theta°$)
Sample 1	Initial	99.4	5.645	99.5	7.280	98.7	3.282
Sample 2	40 °C/75% RH 1 Month	98.7	5.605	98.4	7.039	98.3	3.248
Sample 3	40 °C/75% RH 2 Months	98.2	5.576	97.7	6.885	98.0	3.223
Sample 4	25 °C/60% RH 1 Month	99.6	5.657	99.1	7.192	99.3	3.332
Sample 5	25 °C/60% RH 2 Months	99.0	5.622	98.5	7.061	98.8	3.290

Analysis of variance (ANOVA) table for the regression is constructed and is represented in Table 5. The sum of squares terms (SS) and the regression term represents different sources of variability in the calibration data that can be accounted by the fitted regression line. The SS values for ABC, 3TC and NVP are 12.78769363, 183.535355 and 26.20613828 respectively. Since these values are large we conclude that there is a good linear relationship and the fitted line will describe majority of variability in response with concentration and this is also confirmed by the low residual values i.e., 0.023471102 for ABC, 0.402378604 for 3TC and 0.04263845 for NVP. The mean square term is the ratio of SS term to its degree of freedom (*df*) and the F value (which is the ratio of the regression MS term to the residual MS term) for ABC, 3TC and NVP are found to be 4903.444352, 4105.134271 and 5531.515396
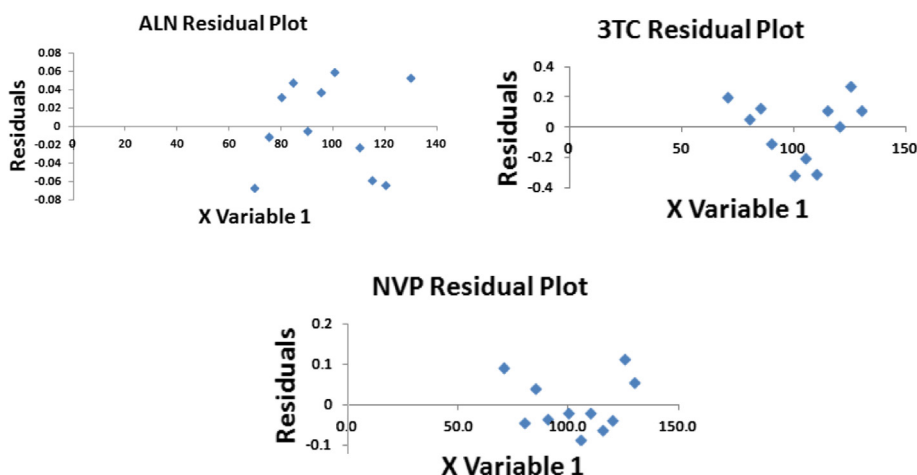

Fig. 5. Residual plots of ABC, 3TC and NVP.

respectively. The larger F values and the larger values of regression MS term compared to residual MS term indicate the good linear relationship. The significance F values (*p*-values) for ABC, 3TC and NVP are found to be 1.24841E-13, 2.77336E-14 and 7.26413E-15 respectively. The extreme small significance F values for ABC, 3TC and NVP indicate that the results were unlikely to have happened by chance representing the strong relationship between data values. The standard error for each coefficient of ABC, 3TC and NVP are found to be 0.000825718, 0.003417904 and 0.001118586 respectively. The lower and upper confidence limits for the gradient and intercept at confidence level 95% for ABC, 3TC and NVP are given in Table 5. These represent the extremes of the values that the gradient and intercept could take. Since the *t*-stat value is larger number and *p*-value greater than 0.05 for regression at 95% confidence level we can say that they are statistically significantly different from zero.

In order to assess the validity of the developed XRPD method it has been applied in the determination of crystalline content of drug formulation of unknown compositions initially and kept at different stability conditions. The results are evaluated from their respective calibration models. The results obtained are tabulated in Table 6. From the results obtained we conclude that the proposed method can be applied easily to analyse large number of samples to determine crystallinity content of drug product. Ideal residual plots are obtained for ABC, 3TC and NVP and are scattered randomly around zero Fig. 5.

4. Conclusion

Powder X-ray diffraction can be a viable method to monitor % crystallinity stability of the active pharmaceutical ingredient in tablet formulations. Here it is shown that XRD measurements of the drug formulations under study were able to detect crystallinity content truly. Regression statistics and ANOVA table results demonstrates the adequacy of the method.

Conflicts of interest

All authors have none to declare.

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