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Development and Validation of Bio Analytical HPLC method for the estimation of Dienogest in Bulk and Pharmaceutical Drugs in Rat Plasma

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Abstract: *A simple, rapid, precise, sensitive and reproducible reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for the bio analytical method for Dienogest with Norethisterone as Internal Standard in pharmaceutical dosage form. Chromatographic separation of Dienogest was achieved on Waters Alliance-e2695, by using Waters Symmetry C_{18}, 150x4.6mm, 3.5µm column and the mobile phase containing 0.1% OPA & ACN in the ratio of* ***40:60% v/v****. The flow rate was 1.0 ml/min; detection was carried out by absorption at* ***214nm*** *using a photodiode array detector at ambient temperature. The number of theoretical plates and tailing factor for Dienogest were NLT 2000 and should not more than* ***2*** *respectively. % Relative standard deviation of peak areas of all measurements always less than* ***2.0.*** *The proposed method was Bio-analytical validated according to USFDA guidelines. The method was found to be simple, economical, suitable, precise, accurate & stable method for pharmakinetics analysis of Dienogest and study of its stability.*

Key Words: *HPLC, Dienogest, Norethisterone, Rat Plasma, Bio Analytical.*

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I. Introduction

Historically, the initial drivers to measure the presence of drugs in biological fluids were to determine possible overdosing as part of the new science of forensic medicine/toxicology. The need to measure drug levels in biological fluids was further driven by the development of pharmacokinetics as a science in the 1930s.

In the early days of drug[1] development, many of the assays for drugs in biological fluids were nonspecific and did not discriminate between the drug and its metabolites; for example, aspirin (circa 1900) and sulfonamides (developed in the 1930s) were quantified by the use of colorimetric assays, while antibiotics could be quantified by their ability to inhibit bacterial growth. The 1930s also saw the rise of pharmacokinetics – a driver for more specific assays.

The lack of specificity became a real issue as the identification of metabolites gathered speed for drugs in biological fluids. Their development as drugs[2] in their own right made identification a commercial issue, as metabolites could be patented as 'new' drug entities. An understanding of the impact of the body's metabolism of drugs became essential for identifying the therapeutically active moiety, as well as possible 'toxic' metabolites[3]. The development of chromatographic techniques such as paper chromatography took place in the 1940s and allowed separation of the drug from its metabolites[4]. Later in the 1950s, thin-layer chromatography[5] was developed and used to quantify drugs in biological fluids, although its main application was in the separation of radio labeled metabolites. Unfortunately, the sensitivity of these technologies was not sufficient to measure the new drugs[6] of the 1950s, such as 'tricycles' that had levels of ng/ml[7].

1.1 Application of a Validated Bioanalytical Method to Routine Drug Analysis

It should be reminded that the effort of a method validation is undertaken to guarantee during the routine analysis a quality of the measurement data as needed for the application for bioavailability, bioequivalence or pharmacokinetic studies such as preclinical pharmacokinetic studies, preclinical toxicokinatic study, clinical toxicokinatic study regulatory toxicokinatic study etc. The different pharmacokinetic and bioequivalence studies require such validated bioanalitical methods, which meet the international rules and the selective and specific determination of the compound, the internal standard and metabolites. Pharmacokinetics describes the absorption, distribution, metabolism and elimination of drugs. Pharmacokinetic studies are important in the generic drug development, when the mentioned safety studies are not necessary to execute. Substitutability of the generic and original formulations is proved bioequivalence studies. The biological equivalence is investigated by studying the statistical accordance of pharmacokinetic parameters.

1.2 Purpose of Bioavailability Studies

Bioavailability studies are performed for both approved active drug ingredients and therapeutic moieties not yet approved for marketing by the drug approving Regulatory Authority. New formulations of active drug ingredients must be approved by that authority before marketing. In approving a drug product for marketing, the Regulatory Authority ensures that the drug product is safe and effective for its labeled indications for use. Moreover, the drug product must meet all applicable standards of identity, strength, quality, and purity. To ensure that these standards are met, the Regulatory Authority requires bioavailability/pharmacokinetic studies and, where necessary, bioequivalence studies for all drug products (FDA Guidance for Industry, 2003)[21]. Bioavailability may be considered as one aspect of drug product quality that links *in-vivo* performance of the drug product used in clinical trials to studies demonstrating evidence of safety and efficacy.

For un-marketed drugs that do not have full NDA approval by the FDA, *in-vitro* and/or *in- vivo* bioequivalence studies must be performed on the drug formulation proposed for marketing as a generic drug product. Furthermore, the essential pharmacokinetics of the active drug ingredient or therapeutic moiety must be characterized. Essential pharmacokinetic parameters, including the rate and extent of systemic absorption, elimination half-life, and rates of excretion and metabolism, should be established after single- and multiple-dose administration. Data from these in-vivo bioavailability studies are important to establish recommended dosage regimens and to support drug labeling.

The aim of the work is to develop and validate bio analytical HPLC method for the estimation of Dienogest in bulk and pharmaceutical drugs in rat plasma.

II. Drug Profile

DIENOGEST

2.1 Structure of Dienogest:

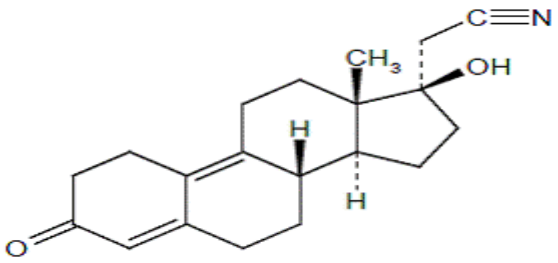

Fig 1: Structure for Dienogest

2.2 IUPAC Name: 2-[(8*S*,13*S*,14*S*,17*R*)-17-hydroxy-13-methyl-3-oxo-1,2,6,7,8,11,12,14,15,16-decahydrocyclopenta[a]phenanthren-17-yl]acetonitrile

2.3 Molecular weight: 311.425g/mol

2.4 Molecular formula: $C_{20}H_{25}NO_2$

2.5 Category: Dienogest, sold under the brand names Natazia and Qlaira among others, is a progestin medication which is used in birth control pills and in the treatment of endometriosis. It is also used in menopausal hormone therapy and to treat heavy periods. Dienogest is available both alone and in combination with estrogens.

2.6 Mechanism of Action of Dienogest: Dienogest acts as an agonist at the progesterone receptor (PR) with weak affinity that is comparable to that of progesterone but has a very potent progestagenic effect in the endometrium, causing endometrial atrophy after prolonged use . It promotes antiproliferative, immunologic and antiangiogenic effects on endometrial tissue. Dienogest reduces the level of endogenous production of oestradiol and thereby suppressing the trophic effects of oestradiol on both the eutopic and ectopic endometrium. Continous administration of dienogest results in hyperprogestogenic and moderately hypoestrogenic endocrine environment, which causes initial decidualization of endometrial tissue. It is an antagonist at androgen receptors, improve androgenic symptoms such as acne and hirsutism.

III. Experimental work

3.1 Chromatographic conditions:

During the selection of chromatographic conditions, number of trails were carried out and the best trail was selected for optimized method.

3.1.1 Preparation of Dienogest stock solution

The stock solution of Dienogest used during HPLC method development stage was prepared by dissolving the accurately weighted standard compound in acetonitrile. Concentration of Dienogest stock solution was 1 mg/ml. Appropriate dilutions with mobile phase were made from the stock solution to prepare the working standard solutions for method development, calibration curve and quality control (QC) samples. The solution and working standard solutions were stored in polypropylene vials in a -20 °C freezer.

3.1.2 HPLC method development of Dienogest

A robust, selective and sensitive HPLC Method with UV detection was developed to quantify Dienogest in rat plasma. It involved evaluation and optimization of the various parameters like sample preparation, chromatographic separation, detection and quantification. Steps involved in method developments are mentioned below in the order as they were followed.

3.1.3 λ_{max} determination of Dienogest:

A stock solution containing 1 mg/ml of Dienogest was prepared by dissolving the drug in acetonitrile. This stock solution was further diluted to 10µg/ml with acetonitrile. Aliquots of this solution were taken HPLC vial and scanned for λ_{max} PDA Detector within the wavelength region of 200–400 nm. . The absorption curve shows isobestic point at 214nm. Thus 214 nm was selected as detector wavelength for the HPLC chromatographic method.

3.1.4 Selection of buffer in the mobile phase

A 0.1% OPA buffer based mobile phase was first investigated to develop a new H P L C method with UV detection. Different buffers with mobile phase conditions are noted and also tried and the chromatograms obtained were compared to find out most suitable buffer in the mobile phase for the proposed HPLC method.

Particulate materials in chemicals used to prepare buffers have been reported as the potential source of many problems with HPLC operation. These materials generally block the solvent reservoir inlet filters, piston, column inlet frits, and thereby reduce the life of column. Therefore, particulates must be removed from the buffer prior to use in the mobile phase. In order to avoid that problem, all the mobile phases after mixing the buffers with organic solvents were filtered with 0.45µ membrane filter.

3.1.5 Selection of Mobile Phase: Based on the trial and error method ratio of the buffer and organic (acetonitrile) was optimized. Each of the buffer systems mentioned in previous section was mixed with Acetonitrile.

After equilibrating of the system for at least 30minutes, Dienogest 10µg/ml was injected. The run time was set for 30 mints initially to confirm the peak followed by minimum run time to avoid the unnecessary wastage of mobile phase. Selection of best mobile phase was based on peak shape, retention time, theoretical plate count, asymmetry factor, resolution etc.

3.1.6 Selection of the Stationary phase (column) for the Dienogest

After selection of proper mobile phase, HPLC column was selected again by trial and error method. Bioanalytical HPLC method differs from simple HPLC method meant for analysis of raw drug. The matrix used in bioanalysis gives the trouble to HPLC analyst as the matrix compounds also mostly co-elute with analyte. To separate the interfering peak from the analyte, different stationary phases like C18, C8, Cyano etc were tried. All other chromatographic conditions except the column were remained fixed during the entire procedure.

3.1.7 Selection of the internal standard (IS) for Dienogest

One of the most important parts of analysis in bioanalytical method is internal standard (IS). As thumb rule, a compound with structural similarity with the analyte or with significant absorbance at the detection wavelength is selected as IS in bioanalytical HPLC method. Good extraction recovery and or chromatographic behavior similar to the analyte would be added advantage. Norethisterone similarity to the analyte were tested as IS for Dienogest HPLC method.

3.1.8 Optimization of the final mobile phase

After selection of the internal standard, final tuning with mobile phase composition and buffer concentration was done based on the retention time of the Dienogest and IS. The final mobile phase was selected so that it could elute the Dienogest and IS with reasonable peak separation.

3.1.9 Optimization of the flow rate

After optimization of mobile phase composition, different flow rates are experimented to ensure proper RT, peak asymmetry and resolution for both drug and IS. From this the finalized flow rate is selected depending on the RT, proper peak asymmetry and resolution.

3.2 Extraction of Dienogest from plasma sample

Plasma sample as such cannot be injected onto HPLC system to quantify the drug. Then it will bock the HPLC column and make it unusable further. Before sample analysis, drug has to extract in suitable solvent followed by its evaporation to concentrate it prior to injection onto HPLC system.

3.2.1Sample extraction procedure

200 µl of blank plasma
↓
500 µl of ACN
↓
Add 500 µl of Dienogest
(10µg/ml)
↓
Add 500 µl of I.S (20 µg/ml)
↓
Add 1300 µl of Diluent
↓
Vortex for 5mins
↓
Centrifuge for 20min at 5000rpm
↓
3ml supernatantant was seperated
↓
Inject the sample on the HPLC

3.2.2Selection of extraction solvent

Simple liquid-liquid extraction was not reported in literature for Dienogest 200 µl blank rat plasma was taken and spiked with drug (10µg/ml) and IS (20µg/ml). Extracted with different organic solvents like dichloromethane; ethyl acetate, chloroform, chloroform: dichloromethane, Chloroform: isoamyle alcohol; Chloroform; isopropyl alcohol, Acetonitrile. Depending on the reproducibility and higher level of recovery, extraction solvent was selected.

3.2.3 Estimating LOD and LLOQ (Signal- to-noise method)

By using the signal-to-noise method, the signal- to-noise ratio, around the analyte retention time was measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio was estimated. The noise value was calculated based on the peak height of the blank plasma around the retention time of Dienogest. The noise magnitude was measured either manually on the chromatogram printout. Generally The analyte amount for which the signal- to-noise ratio was equal or more than 3 times was identified as LOD LLOQ was determined by the analyte amount for which the signal-to-noise ratio was equal or more than 5 times.

IV. Results And Discussion

4. 1 Development of HPLC method for Dienogest

4. 1.1 Determination of λ_{max} for Dienogest

Isosbestic point (λ_{max}) of Dienogest was found to be a t 214 nm. It indicates that detection at 214 nm would be the most sensitive wavelength for HPLC work. This λ_{max} was selected for HPLC method development of Dienogest.

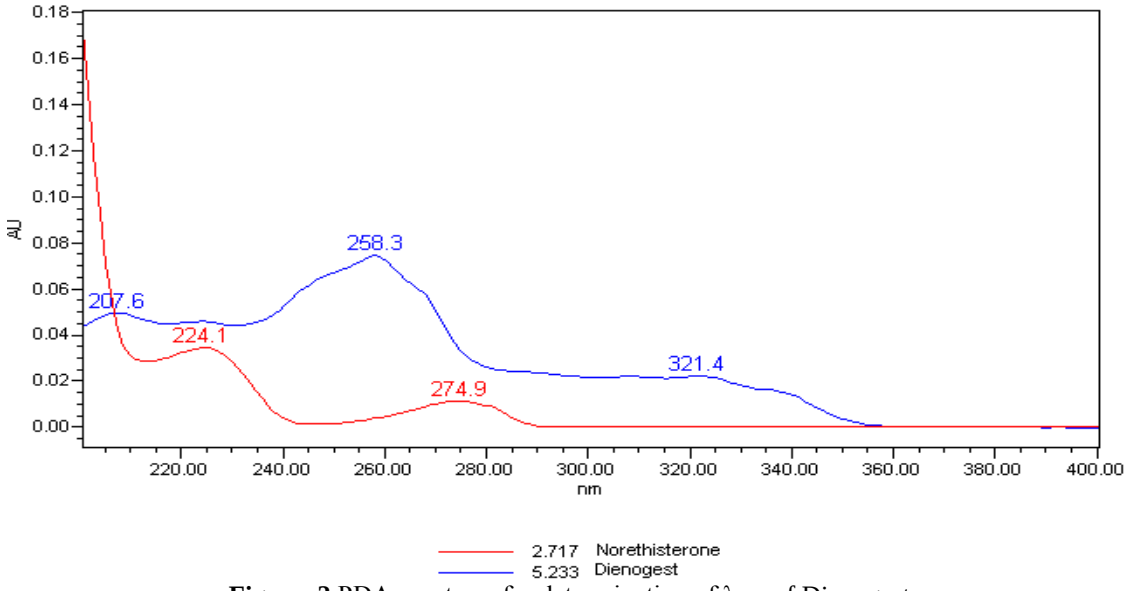

Figure. 2 PDA spectrum for determination of λ_{max} of Dienogest

4.1.2 Analytical Method Development of Dienogest By RP-HPLC Method

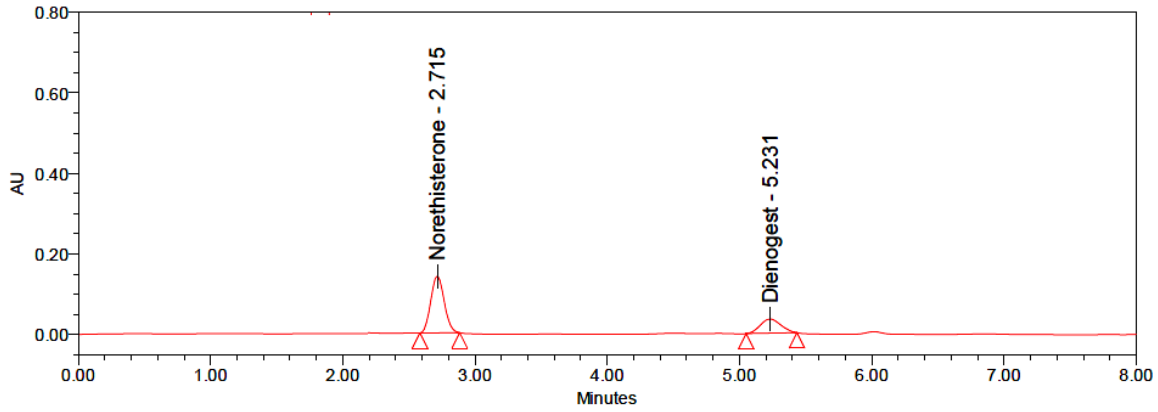

Figure. 3 Chromatogram

	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1	2.715	18532	65.98		1.02	3274
2	5.231	10246	20.31	9.88	1.11	5131

Table: 1 Dienogest and IS

4.2 Method validation for bioanalytical studies of Dienogest

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation.

4.2.1 Specificity and Selectivity

No interfering peaks were found in six different random blank rat plasma samples at the retention times of either Dienogest or ISTD.

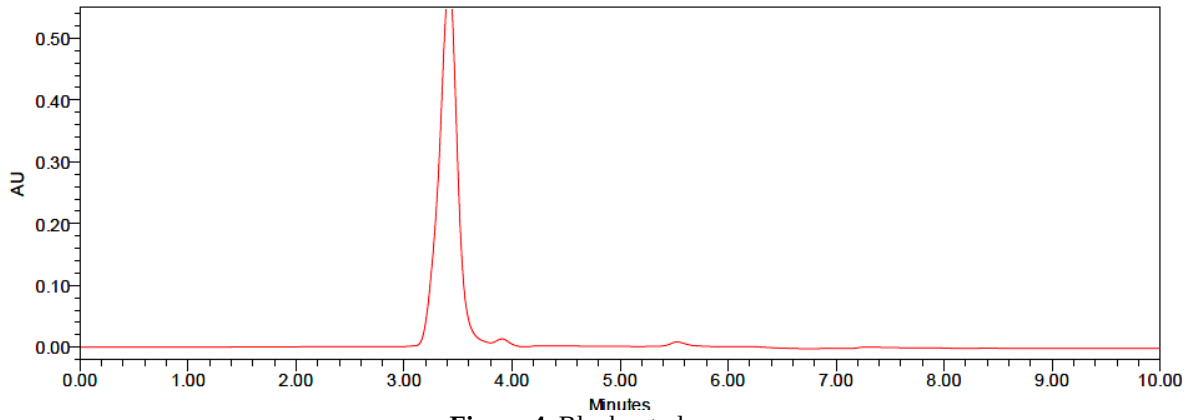

Figure.4 Blank rat plasma

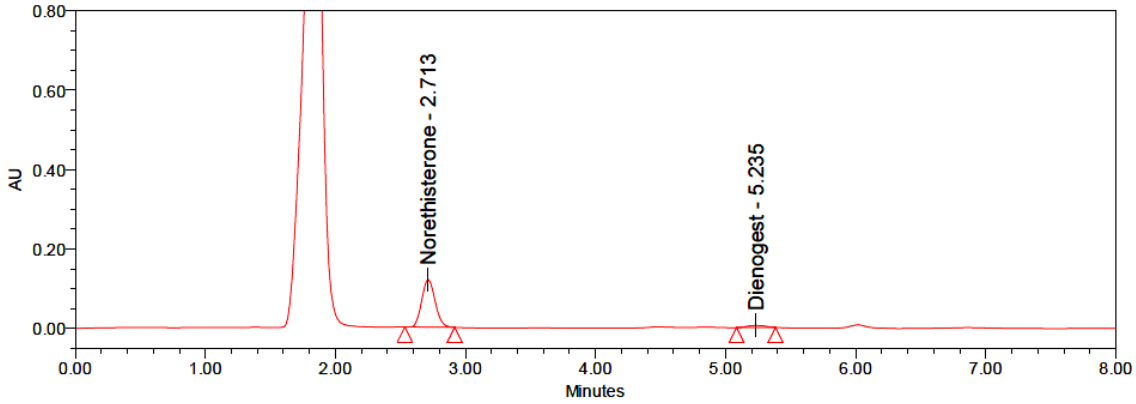

Figure 5 Blank rat plasma spiked with analyte at LLOQ and IS

As observed from the above chromatogram, total run time was 10 min and the retention time of drug and IS where about 5.235 and 2.713min respectively. For blank plasma chromatogram there was no interfering peaks nearthe peaks for Dienogest and IS. Same is observed in case of the chromatogram of blank plasma spiked with IS.

4.2.2 **System suitability:** The %RSD for Dienogest and istd area ratio was found to be 1.53%. Hence it passed the system suitability.

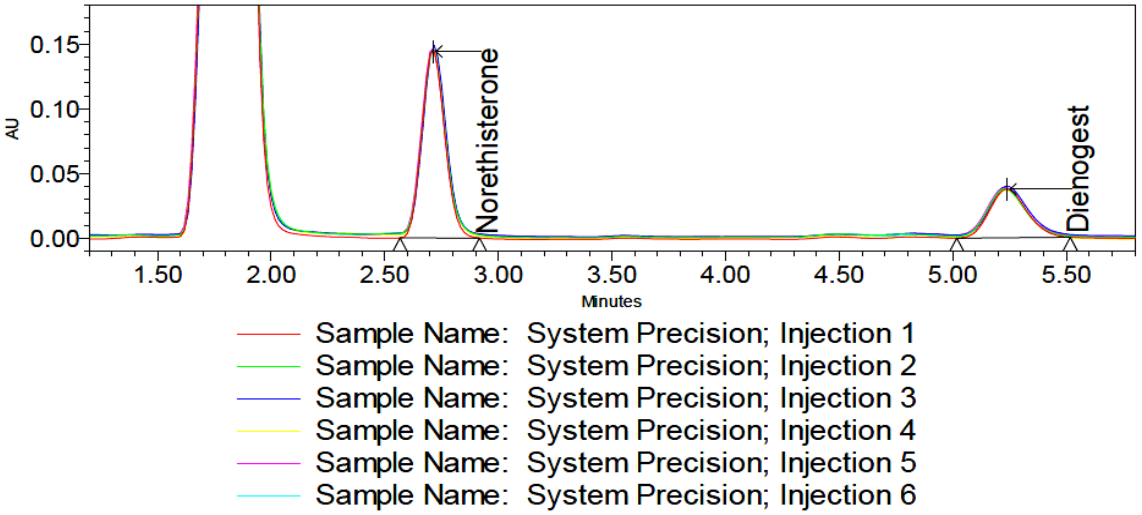

Figure No 6 Overlay chromatogram of system suitability

4.2.3 **Sensitivity:** The %RSD for Dienogest and istd area ratio was found to be 1.33%. Hence it passed the sensitivity.

Table: 2 Sensitivity Results of Dienogest

Replicate Number	LLOQ Nominal Concentration(µg/ml) 0.5357 Nominal Concentration Range(µg/ml) (0.5264-0.5465) Calculated Concentration(µg/ml)
1	0.5268
2	0.5297
3	0.5346
4	0.5355
5	0.5452
6	0.5426
N	6
Mean	0.5357
SD	0.00713
%RSD	1.33
% Mean Accuracy	98.36%

4.2.4 Linearity:

The standard curves were linear over the concentration range of 5-20 µg/ml of Dienogest. The mean correlation coefficient was 0.999. Samples were quantified using the ratio of peak area of analyte to that of IS. Peak area ratios were plotted against plasma concentrations.

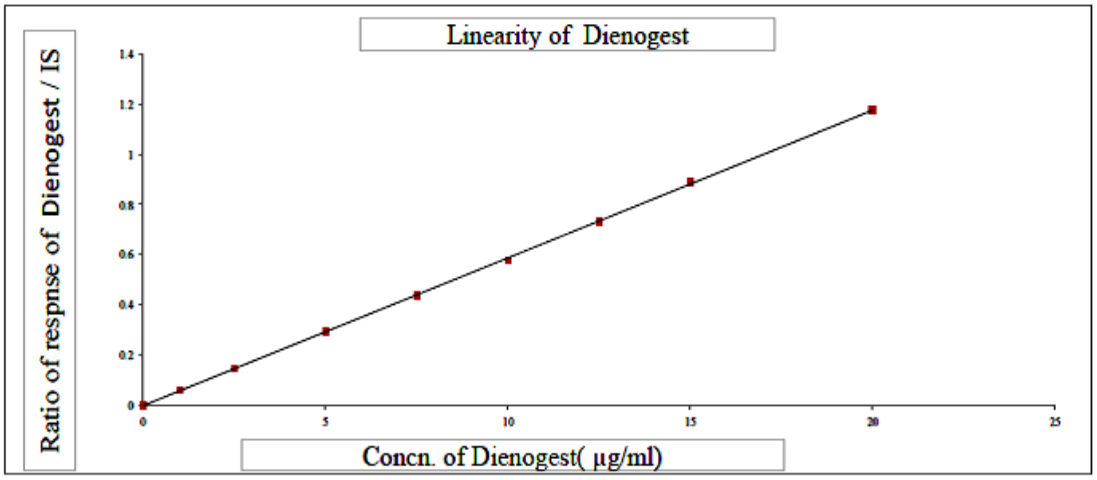

Figure 7 Calibration plot for concentration v/s Area ratio of Dienogest

4.2.5 LOD and LOQ

LOD and LOQ were separately determined by calibration curve method. LOD and LOQ of the compound were determined by injecting progressively lower concentrations of standard solutions using developed RP-HPLC method. The LOD concentrations for Dienogest is 0.1 µg/ml their s/n values are 4. The LOQ concentration for Dienogest is 1.0 µg/ml their s/n values are 24.

Table 3: LOD and LOQ data for Dienogest

Name	**LOD**		**LOQ**	
	Concentration (µg/ml)	**s/n**	**Concentration** (µg/ml)	**s/n**
Dienogest	0.10	4	1.0	24

4.2.6 Precision and accuracy: The intra-assay precision and accuracy were estimated by analyzing six replicates containing Dienogest at six different QC levels. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data include, accuracy within 85–115% from the actual values and a precision of within ±15% relative standard deviation (RSD) except for LLQC, where it should be within 80–120% for accuracy and <20% of RSD

Table 4: Accuracy and precision of data of the Dienogest (n= 6)

Quality control sample	Spiked concentration (µg/ml)	Mean (µg/ml)	SD	Accuracy (%)	RSD (%)
Intra-day					
LLOQ	0.5263	0.5257	0.00712	97.32	1.35
LQC	5.1672	5.1657	0.10754	99.28	2.08
MQC	10.2697	10.2364	0.09762	100.05	0.95
HQC	15.1364	15.1472	0.25314	99.89	1.67
Inter-day					
LLOQ	0.5251	0.5266	0.00718	98.85	1.33
LQC	5.1674	5.1634	0.10462	97.66	1.95
MQC	10.2648	10.2653	0.0957	99.67	0.92
HQC	15.1341	15.1362	0.25634	96.36	1.57

4.3 Stability:

4.3.1 Stability on day zero: The %CV and mean accuracy for Dienogest were found to be 1.23%, 1.31%. Hence it passed the Stability on day zero.

Table 5: Stability on day zero of Dienogest

Replicate No.	HQC	LQC
	Nominal Concentration(µg/ml)	
	15.454	5.364
	Nominal Concentration Range(µg/ml)	
	(15.123-15.678)	(5.264-5.548)
	Back Calculated Concentration(µg/ml)	
1	15.136	5.264
2	15.348	5.347
3	15.546	5.328
4	15.559	5.459
5	15.672	5.427
6	15.461	5.358
N	6	6
Mean	15.454	5.364
SD	0.189	0.070
% RSD	1.23	1.31
% Mean Accuracy	98.16%	97.88%

4.3.2 Wet Extract: The %RSD and mean accuracy for Dienogest were found to be 0.42%, 0.10%, 0.40%, 0.27%. Hence it passed the Wet extract at -28◦C.

Table 6: Wet Extract of Dienogest

Replicate No.	HQC		LQC	
	Nominal Concentration (µg/ml)			
	15.537	15.502	5.549	5.534
	Nominal Concentration Range (µg/ml)			
	(15.458-15.698)	(15.478-15.525)	(5.512-5.577)	(5.501-5.551)
	Back Calculated Concentration (µg/ml)			
	Compaison samples	Stability samples	Compaison samples	Stability samples
1	15.462	15.487	5.524	5.540
2	15.495	15.496	5.529	5.549
3	15.526	15.501	5.534	5.518
4	15.507	15.517	5.575	5.512
5	15.604	15.524	5.562	5.538
6	15.627	15.486	5.569	5.545
n	6	6	6	6
Mean	15.537	15.502	5.549	5.534
SD	0.06481	0.01566	0.02234	0.01508
% RSD	0.42	0.10	0.40	0.27
% Mean Accuracy	99.46%	98.28%	98.55%	98.23%
% Mean Stability	98.37%		98.39%	

4.3.3 Freezetahaw at -80◦C: The %RSD and mean accuracy for Dienogest were found to be 0.83%, 0.39% and 2.76%, 2.35%. Hence it passed the Freezetahaw at -80◦C.

Table 7: Freezetahaw at -80◦C of Dienogest

Replicate No.	HQC		LQC	
	Nominal Concentration (µg/ml)			
	15.159	15.628	5.436	5.629
	Nominal Concentration Range (µg/ml)			
	(15.012-15.489)	(15.542-15.752)	(5.231-5.655)	(5.457-5.854)
	Back Calculated Concentration (µg/ml)			
	Compaison samples	Stability samples	Compaison samples	Stability samples
1	15.017	15.546	5.234	5.486
2	15.026	15.578	5.364	5.526
3	15.157	15.624	5.347	5.578
4	15.169	15.659	5.469	5.621
5	15.234	15.642	5.564	5.723
6	15.348	15.721	5.639	5.841
n	6	6	6	6
Mean	15.159	15.628	5.436	5.629
SD	0.126	0.062	0.150	0.132
% RSD	0.83	0.39	2.76	2.35
% Mean Accuracy	99.11%	99.45%	99.02%	99.43%
% Mean Stability	99.28%		99.24%	

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