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RP-HPLC method to assay ethinylestradiol and drospirenone with 3D chromatograph model in fixed dose in rat plasma for pharmacokinetic study analysis

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Abstract: For the simultaneous measurement of Ethinylestradiol (EE) and Drospirenone (DP) in fixed-dose combination hormones tablets, a reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed. A specific, precise and accurate RP-HPLC method was developed and validated to analyse the drugs in rat plasma. The fluorescence detection for EE was made at λ= 200-310 nm and Ultraviolet–visible (UV/Vis) detection for DP was made at 270 nm. The typical EE and DP retention times were 4.19 and 5.30 minutes, respectively. The limit of detection (LOD) and limit of detection (LOQ) for EE were 0.121 and 0.282µg/mL and LOD and LOQ for DP were 2.23 and 7.697µg/mL respectively. The regression coefficient (r^2) of EE and DP were 0.9937 and 0.9913 respectively. Precision's relative standard deviation (RSD) was less than 5%. The analyte recoveries of both drugs stayed within 95% of each other. All other validation parameters adhered to ICH standards. Throughout the analytical process, the analyte was stable. The advantages of the method developed include stability under different conditions and a low limit of quantification that was in micrograms. Its applicability was confirmed by the analysis of EE and DP levels in plasma samples in a designed pharmacokinetic study in rats after oral administration.

Keywords: ICH guidelines, RP-HPLC, simultaneous determination, Ethinylestradiol (EE) and Drospirenone (DP).

INTRODUCTION

Estrogen and progestin work together to efficiently avert ovulation, which is the fundamental goal of contraception. Recent advancements in oral contraceptives have focused on the development of novel formulations with fewer side effects and benefits beyond contraception, thus assuring wider adherence to contraception methods. To guarantee the balance between efficacy, safety and cycle control, new formulations have been developed by correctly combining low dosages of EE and DP (Gupta *et al*., 2022, Regidor *et al*., 2023). Quality is a crucial component of this type of formulation due to the modest doses utilized and the prolonged hormonal treatment. For the quantitative determination of associated hormones in various contraceptive formulations, several high-performance liquid chromatographies (HPLC) methods have been described in the literature (Hameedat *et al*., 2022, Rajeswari *et al*., 2019). However, no analytical technique for the simultaneous quantitative measurement of EE and DP in plasma has been explored to date. The current study outlines an easy, accurate and trustworthy validated analytical approach for EE and DP in plasma, simultaneous measurement for coated tablets employing RP-HPLC with UV and fluorescence detection. The technique can be applied to routine quality control analysis of these pharmaceutical compositions. Nevertheless, to the best of our knowledge, simple, inexpensive and robust pharmacokinetic analytical methods with low limits of quantification and excellent accuracy for simultaneous estimation of EE and DP in biological samples have not been reported so far.

Progression and approval of the current RP-HPLC method in accordance with the guidelines of ICH are helpful in more reliable simultaneous quantification of EE and DP in pharmaceutical formulations and dissolution tests and so on. Moreover, this proposed method with high sensitivity will be particularly helpful for those laboratories that do not have more sophisticated equipment, such as GCMS, LCMS and so on, for quantification purposes. In this scenario, this study aimed to develop and validate an analytical LC-UV method based on a liquid-liquid technique to extract the drug from rat plasma. This approach was proposed to afford low limits of quantification to assay in rat plasma, considering the requirements for suitable application in a pharmacokinetic study after oral administration.

MATERIALS AND METHOD

Ethinylestradiol (EE) and Drospirenone (DP) (Saffron Pvt Ltd., Lahore and Punjab, Pakistan) were the presented samples. The tablet (Yasmin tablet, Bayer

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Pharmaceuticals Ltd) containing 0.02mg of EE and 3mg of DP was obtained from the company. Daejung Chemical Co. (Siheung, South Korea) was the hub to acquire purified grade ammonium acetate, methanol, HPLC grade acetonitrile and potassium dihydrogen phosphate. The remaining reagents employed in this research were of analytical grade. Calibrated analytical balance was used for weighing purposes. Calibrated glassware was used throughout the operation. Double distilled water was used in the RP-HPLC method.

Instrumentation and analytical conditions

Chromatographic studies were carried out using Shimadzu RP-HPLC equipment (Shimadzu Corporation, Kyoto, Japan), which has quaternary LC-20AD pumps outfitted with a DGU-20A3 degasser and a variable wavelength programmable UV visible/PDA/florescent detector SPD-20A. Bonna-Agela technologies (Tianjin, China) supplied the Promosil C18 series column (250 mm 4.6 mm, 5µm), which was used for analysis. Ammonium acetate buffer (pH = 7.0), acetonitrile and methanol (20:40:40, v/v/v) made up the mobile phase. The mobile phase flow rate for isocratic elution was 1.5mL min. The operation involved the use of a Shimadzu AW220 electronic balance, a James ultrasonic bath. The injection had a 20µLvolume.

Preparation of standard stock solution

Standard solutions for both drugs were prepared by serial dilution from a solution of 50.0µg/mL^{-1} with the mobile phase to obtain analyte concentrations of EE (1.20, 2.40, 3.60, 4.80 and 6.00µg/mL) DP (120, 240, 360, 480 and 600µg/mL). The internal standard working solution was prepared in methanol to yield a concentration of 0.50 mg/mL and diluted to 10.0µg/mL^{-1} immediately before use.

Standard solutions in plasma

100 µL of blank rat plasma, 900µL of EE and DP standard solutions were used to generate the samples for the analytical curves EE (1.20, 2.40, 3.60, 4.80 and 6.00 µg/mL) DP (120, 240, 360, 480 and 600µg/mL). The internal standard's final concentration was 10µL. The drug was removed from the plasma using the liquid-liquid method. The standard solution in an Eppendorf tube was mixed with ethanol for precipitation and the mixture was vortexed for 5 minutes. The samples were centrifuged (6000g for 10min at 4°C) after being shaken for 10 minutes. The supernatants were moved to tubes and centrifuged at 40°C to allow the organic solvent to evaporate. The pellet were resuspended in the mobile phase (1000µL), vortex mixed for 5 seconds, sonicated for 10 minutes and then assayed using the RP-HPLC method.

Validation of bioanalytical methods

According to FDA Guidance - Bioanalytical Method Validation (2001), the method was validated by taking into account linearity, the lower limit of quantification, specificity, precision, accuracy and stability (Girme *et al*., 2021, Kaza *et al*., 2019, Magar and Shakil, 2020).

Linearity, lower limit of quantitation and limit of detection

Four analytical curves with plasma concentrations ranging from EE (1.20, 2.40, 3.60, 4.80 and 6.00µg/mL^{-1}) DP (120, 240, 360, 480 and 600µg/mL^{-1}) for both drugs were measured on two consecutive days in order to test linearity. According to the procedure outlined in the "Sample preparation" section, the drug was removed from the biological samples and dissolved in 1000µL of the mobile phase. This was done before the RP-HPLC analysis. The average relative area of each concentration of the curve was used to plot each analytical curve. To get the equation of the calibration curve and correlation coefficients, linear regression was used to examine the four concentrations of the standard solution. The limit of quantification was defined as the lowest concentration in the analytical curve with acceptable precision and accuracy.

Accuracy and precision

In six replicate analyses, the standard deviation (SD) and relative standard deviation (RSD %) of three concentrations (low, medium and high) were calculated in order to assess the intra and inter-day precision as well as the accuracy of the analytical method. As previously mentioned, the medication was extracted and then suspended in 100µL of the mobile phase to achieve concentrations of 1.20-3.60-6.0µg of EE and 120-360-600µg of DP (Dalvi *et al*., 2018).

Recovery

The analytical results of extracted samples from three concentrations of 1.20-3.60-6.0µg of EE and 120-360-600µg of DP were compared with those obtained from a standard solution, which is 100% of the recovery, to determine relative recovery (Mehmood *et al*., 2019).

STATISTICAL ANALYSIS

The statistical analysis of different validation parameters were evaluated using IBM SPSS v.20 software package and standard deviation along with percentage determination of the concentration of analyte were calculated.

RESULTS

By choosing a set of parameters in accordance with USP 43 requirements (Ahmed *et al*., 2019), such as a mobile phase made up of acetonitrile, methanol and ammonium acetate buffer (40:40:20%) and a flow rate of 1.5mL/min with column Agela C18, 250mm 4.6mm, 5µm, the appropriateness of the system was evaluated. The

statistical data of various parameters, including retention time (tR), peak area (A), relative standard deviation, symmetry factors (As), theoretical plates (N) and resolution of EE and DP for peak response, were calculated using the Ezichrom chromatography manager program. Table 1's findings describe how the analytical technique was carried out to determine whether the system was suitable and that it complied with USP requirements. The number of theoretical plates exceeded 2000, the capacity factor (K) was 2-10 and the tailing factor was less than 2.0. EE and DP had typical retention periods of roughly 4.1 and 5.3 minutes, respectively and IS peak was at about 6.3 minutes. Endogenous chemicals weren't found until 10 minutes of runtime. Peaks in figs. 3 and 4 showed the relative peak time with rat plasma and without plasma. Plasma peaks can see before 3 minutes.

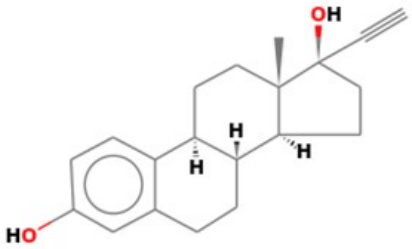

Fig. 1: Chemical structure of Ethinylestradiol (Drug Bank).

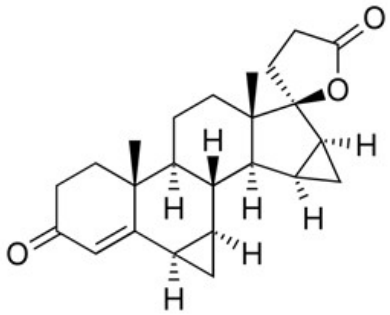

Fig. 2: Chemical structure of Drospirenone (Drug Bank).

A lot of studies have been done on the 3D chromatograms produced by the high Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) in the areas of medicine (Alipanahpour *et al*., 2020, Furusho *et al*., 2019). Most current approaches for separating a 3D chromatogram need prior knowledge of the compound numbers, which can be difficult when the compounds are complicated or there is white noise present. The fig. 4 shows 3D chromatograph of DP (fig. 4) from the back and front. The 3D chromatograms indicate that this method can successfully separate the drug with a fast and effective method.

Linearity, lower limit of quantitation and limit of detection

Four analytical curves with plasma concentrations ranging from EE (1.20, 2.40, 3.60, 4.80 and 6.00µg/mL^{-1}) DP (120, 240, 360, 480 and 600µg/mL^{-1}) for both drugs were measured on two consecutive days in order to test linearity and results were shown in table 1 and 2. The calibration curves obtained by plotting peak area against concentration showed linearity in the concentration range of EE (1.20, 2.40, 3.60, 4.80 and 6.00µg/mL^{-1}) DP (120, 240, 360, 480 and 600µg/mL^{-1}) for both drugs (table 2). Linear regression equations of EE and DP were found to be y= 13.94x+2.66 and y= 30.60x+0.17, respectively and the regression coefficient values (r) were calculated to be 0.9937 and 0.9969, respectively, indicating a high degree of linearity for both drugs (fig. 3 and 4) (table 2). The signal-to-noise ratio method (3:1 for LOD & 10:1 for LOQ) was used to establish the limits of detection and quantitation. Limits of quantification for EE and DP were calculated to be 0.282 and 7.697, respectively, while limits of detection were reported to be 0.121 and 2.230 µg/ml (table 2), demonstrating the proposed method's high sensitivity and suitability for pharmacokinetic and bioequivalence studies that call for the detection of low plasma concentrations.

Accuracy and precision

Table I lists the precision (RSD), accuracy (%) and recovery (%) findings from the analysis of samples containing EE and DP at low, medium and high concentrations of 1.20-3.60-6.0µg of EE and 120-360-600µg of DP respectively. The repeatability (intraday precision) and intermediate precision (inter-day precision), which make up RSD values, were less than 10% and 15%, respectively. The accuracy was close to 100%. According to earlier reports, recovery, which measures extraction effectiveness, was greater than 90% for the three concentrations (Kusuma *et al*., 2021). Additionally, these findings are in accordance with global standards (FDA Guidance - Bioanalytical Method Validation, 2001). Inter-day and intra-day precisions were used to evaluate the accuracy of the developed method. From the results that are reported in table 3, it can be seen that the method is precise. The mean recovery value was 98.393 for EE and 98.696 for DP thus depicting the accuracy of the developed method.

Recovery

The % recoveries of each component at three different concentrations in the ternary mixture were used to determine the accuracy of the devised procedure. Table 4 displays the percentage recovery numbers

DISCUSSION

A method of analysis is crucial to the creation of a drug's product. The easiest and most popular method of drug analysis in the pharmaceutical business is RP-HPLC. The simultaneous determination of sofosbuvir and velpatasvir by RP-HPLC is mentioned in this paper. According to ICH rules, the HPLC method was effectively validated.

Table 1: Results of system suitability parameters

S. No	Parameters	EE	DP
1	Peak Area	2337224± 0.391	971760± 0.273
2	Retention time	4.115± 0.147	5.382± 0.221
3	Theoretical plates	More than 2000	More than 2000
4	Asymmetry	1.70% ± 0.418	1.40% ± 0.246

Table 2: Regression analysis and sensitivity determination along with LOD and LOQ

Parameters	EE	DP
Working λmax	270	200-315
Regression equation	y=23372x	y=901590x
Slop	23372	901590
LOD µg/mL	0.121	2.230
LOQ µg/mL	0.282	7.697
Regression coefficient (r_2)	0.9937	0.9913
Retention time (min)	4.199	5.301

Table 3: Data for intra-day and inter-day precision

Drug	Amt (µg/mL)	Intra-day variation (n=3)		Inter-day variation (n=3)	
EE		% Precision	% RSD	% Precision	% RSD
	1.2	98.231	0.322	99.587	0.251
	3.6	97.212	0.191	98.192	0.652
	6.0	99.032	0.489	97.231	0.389
Average precision		98.158			
DP	120	99.271	0.524	98.763	0.872
	360	97.223	0.293	99.284	0.291
	600	98.384	0.191	99.489	0.104
Average precision		98.292			

Table 4: Results of recovery study by HPLC methods

Drug	Amt. present (µg/mL)	Amt. added (µg/mL)	Amt. found* (µg/mL)	Amt. recovered* (µg/mL)	% Recovery*	% RSD
EE	6.0	2	7.80	1.8	90.00	1.471
	6.0	4	9.85	3.85	96.24	1.083
	6.0	6	11.87	5.87	97.83	1.621
DP	600	50	648	48.0	96.00	1.271
	600	100	689	89.0	89.00	1.203
	600	150	743	143	95.33	1.211

Table 5: Results of stability studies

Drug	Concentration analysed (µg/mL)	Concentration recovered % ± S.D	
		15–25°C, 7 days	2–8°C, 14 days
EE	1.2	99.419± 0.42	98.233 ± 0.21
DP	120	98.652± 0.13	98.162 ± 0.64

The percentage recoveries were found to be satisfied as shown in results of accuracy. The method suitability was ensured by finding tailing factor, number of theoretical plates and peak area. Results shown that theoretical plates numbers (N) were more than 2000 per each peak, tailing factor was less than 1.5 and RSD values for peak areas were less than 2% for both compounds. Stability of suggested method indicated that there is no significant changes were observed on the percentage drugs recovered and their respective RSD values (table 5).

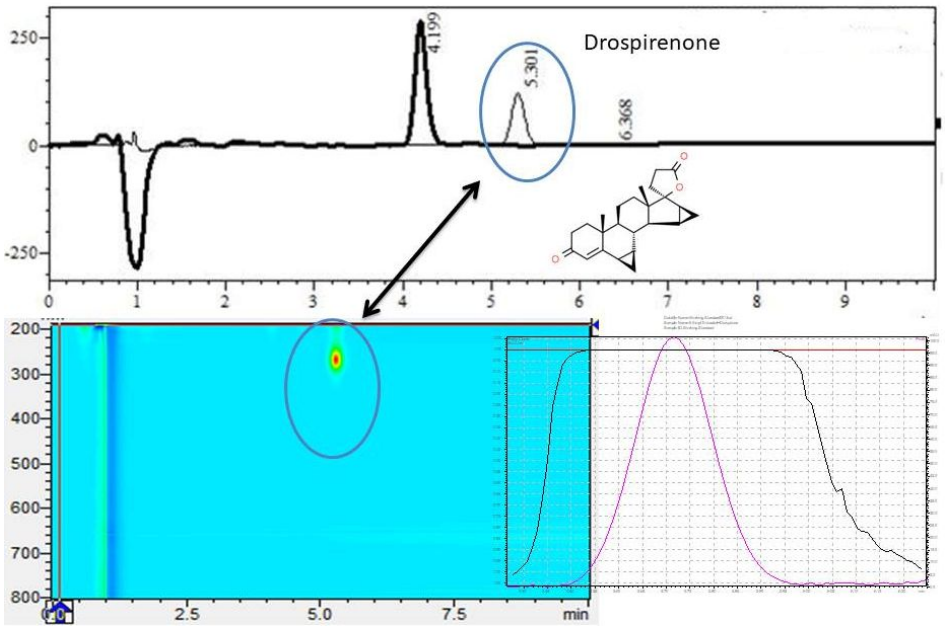

Fig. 3: Chromatogram of DP standards along with colour graph of absorbance

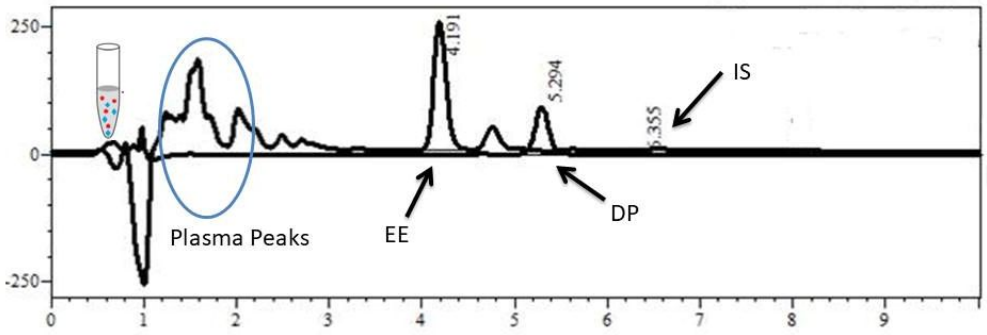

Fig. 4: Chromatogram of EE and DP standards along with rat plasma

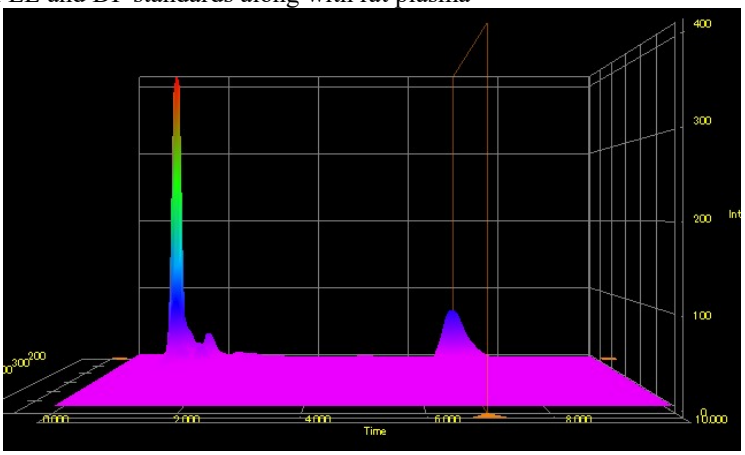

Fig. 5: 3D Chromatogram of DP

The assay that was created was exact, focused, linear and robust. Both the medications in bulk and the dissolving medium may be easily quantified. No interference was found in any sample, proving the assay's applicability. The forced degradation analysis further demonstrates the method's applicability for both qualitative and quantitative assessments of the two medications. The findings of plasma spiking showed that the liquid-liquid extraction method was effective and that the components of rat plasma did not obstruct the separation of the two active moieties. As a result, the new approach is even more appealing because it can quantify pharmaceuticals in both pharmaceutical samples and biological fluids.

CONCLUSION

RP-HPLC method was developed for simultaneous detection and estimation of EE and DP in plasma. The method complied with the guidelines of USP and ICH. The results of the tests complied with both limits and criteria. A method to assay EE and DP in rat plasma for application to pharmacokinetic studies was developed and validated using HPLC-UV/DAD/florescent and a liquid-liquid technique to extract the drug from plasma. This method showed good accuracy, linearity, specificity and precision and was successfully used to analyze rat plasma samples in a pilot pharmacokinetic study. The validated method has notable advantages, like the lower limit of quantitation compared with other HPLC-UV/DAD/ florescent methods previously described in the literature, a good resolution between the analyte and the plasma components and stability under different conditions. The present analytical method is suitable for use in pharmacokinetic studies that require the quantification of very low concentrations of drugs in plasma, such as studies involving the development of innovative formulations containing EE and DP.

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