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Supercritical fluid (carbon dioxide) based ultra performance convergence chromatography for the separation and determination of fulvestrant diastereomers

Ganipisetty Venkata Narasimha Rao,*[a] G. Gnanadev,[a] Bellam Ravi,[a] D. Dhananjaya,[a] P. Manoj,[a] B. Indu[a] and R. Venkata Nadh[b]

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UltraPerformance convergence chromatography (UPC^2 ™) is a new category of separation science which utilizes the unrealized potential of the supercritical chromatography phenomenon. UPC^2™ is a stand-alone, viable technique that is cost effective, sustainable, and uses green technology that lowers the use of organic solvents. Based on this advantage, we explored a simple and robust supercritical liquid-based UPC^2 method in order to increase sample throughput and productivity to quantify the diastereomers of fulvestrant. The two isomers of fulvestrant were well separated on a chiral column (150 mm × 4.6 mm, I.D.) by applying a mixture of methanol and acetonitrile (9.5 : 0.5) as the co-solvent of the mobile phase of carbon dioxide (75%). The detection was carried out at 280 nm. We were able to achieve a three-fold reduction in retention with an isocratic mode as compared to the United States Pharmacopoeias (USP) normal phase method. This new method was validated in accordance with the ICH guidelines; it exhibited good intra- and inter-day accuracy, precision, and the results were linear over a range of 25% to 150% of the target concentration. The method could be successfully applied for the determination of the diastereomeric ratio of fulvestrant as an API and in fulvestrant injectable finished products.

1 Introduction

Today a large amount of hazardous and-non hazardous waste in the form of organic solvents is being generated by various industries. The management of these waste solvents has become a concern in the view of environmental health and safety. There is the need to apply the principles of "green chemistry" in every process at all stages of manufacturing and testing, to reduce the resulting amounts of waste. In this context, supercritical CO_2 has emerged as a versatile solvent for various chemical separations owing to its low toxicity and inflammability. As a result, supercritical fluid chromatography (SFC) has emerged as an analytical tool for chemists for various separations of active compounds and impurities in shorter amounts of time.[1]

In the pharmaceutical industry, SFC is an alternative and complementary method to the HPLC technique. The potential of SFC using packed columns for the analysis of impurities in pharmaceutical compounds has been recognized for many years.[2] SFC can offer highly efficient separations in short analysis times and with a low pressure drop without compromising the resolution, plate counts and tailing. However, the lack of reliable and sensitive commercial SFC systems has prevented the extensive use of SFC in the industry. To explore the 'green' potential of SFC for faster separations, environmental safety and better waste management, an attempt is made here to develop a simple and short method for the determination of fulvestrant diastereomers using UPC^2 (UltraPerformance convergence chromatography).[3]

The main aim of the current study and the future plan are to develop and validate faster and environmentally friendly methods to reduce the solvent consumption and analysis time without compromising any performance parameters such as resolution, peak tailing and plate count by utilizing the green technology SFC. For this, we selected fulvestrant, which is a novel endocrine therapeutic for breast cancer with a unique structure and mode of action. Fulvestrant is the only parenteral agent in this class which has a good side effect report and is well tolerated. Fulvestrant is the subject of much ongoing research regarding its novel mechanism of action and pharmacokinetic profile, to optimize its clinical efficacy and explore new applications, including first-line use in advanced breast cancer.[4–7]

Fulvestrant is commercially available under the name FASLODEX® from Astra Zeneca.

The chemical name of fulvestrant is 7α-[9-(4,4,5,5,5-pentafluoropentylsulphinyl)-nonyl]estra-1,3,5(10)-triene-3,17β-diol. It

[a]Formulations Research and Development Centre, Mylan Laboratories Ltd, Bollaram, Jinnaram, Medak, Hyderabad, Andhra Pradesh, 502325, India. E-mail: narasimha.ganipisetty@mylan.in; Venkata.nr@gmail.com; Tel: +91 9000122344

[b]Department of Biotechnology, Vignan's Engineering College, Vadlamudi, Guntur, Andhra Pradesh, India

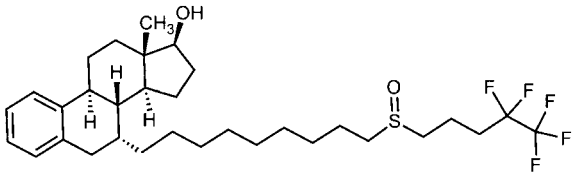


Fig. 1 Chemical structure of fulvestrant.

contains six asymmetric carbon atoms, and a stereogenic sulphoxide in the side chain. The active ingredient is a mixture of two diastereoisomers: fulvestrant sulphoxide A and B, having the same absolute configuration at each of the stereogenic centers in the steroid system, but different absolute configurations at the sulphur atom. The chemical structure of fulvestrant is shown in Fig. 1.[8,9]

Currently, the United States Pharmacopoeia monograph for the fulvestrant drug substance prescribes an isocratic normal phase method for the determination of the diastereomeric ratio.[9] It utilizes a mobile phase consisting of 2-methyl pentane (an aliphatic hydrocarbon) and dehydrated alcohol 880 : 220 at a flow rate of 1 mL min^{-1}. The run time of this method is approximately 30 minutes. It also uses 2-methyl pentane for sample preparation which is a costly affair and a cumbersome process for waste management. USP and current method chromatograms are compared in Fig. 2.

Some literature is available on the analysis of fulvestrant and antiestrogens either by high performance liquid chromatography or HPLC coupled with a mass spectrometry technique.[10–21] One validated high performance liquid chromatography method for the determination of fulvestrant in pharmaceutical dosage forms was reported by Varanasi, which uses normal phase separation on a cyano column using *n*-hexane and isopropyl alcohol as the eluents.[22] The retention times of the two isomers are reported to be around 30 minutes and 30.5 minutes. A difference in retention time of 0.5 minutes between the two peaks indicates a poor resolution. One US patent on the separation of fulvestrant isomers by Cristian Fazioni is available.[23] The patent describes methods for separating the isomers by a reverse phase HPLC and a chiral column using acetonitrile and *n*-hexane as the solvents in the mobile phase. So far, all available literature procedures use one or more solvents described above which many laboratories would like to reduce for health, safety, environmental, and cost reasons. No method has been reported so far on the herein presented detection by SFC. Hence, a green initiative was taken here to develop a method, equivalent or superior to the existing available methods, by using the advantages of SFC to reduce the cost of analysis, and to safeguard the environment.

2 Experimental

2.1 Chemicals and reagents

Samples of the fulvestrant API and injectable material used in this study were obtained from the Mylan R&D Center (Hyderabad, India). The reference standard used was an in-house generated standard from Mylan. The HPLC grade acetonitrile and methanol were obtained from Merck, India. The CO_2 was purchased from Sai Padmaja Oxygen (Hyderabad, India).

2.2 Instruments and chromatographic conditions

An integrated Acquity UPC^2 system from Waters Corporation, Milford, USA, was equipped with a Waters photodiode array detector (PDA). Data collection and analysis were performed using the Empower software 2pro (Waters Corporation). Balances used for weighing the reference standards and samples were from Mettler Toledo.

Separation was achieved on a Chiralpak AD-H (Diacel) column with the dimensions 150 mm × 4.6 mm (I.D.) and a particle size of 5 μm. A simple mobile phase containing liquid CO_2 and mixtures of methanol and acetonitrile with the ratio 95 : 5 (v/v) as the co-solvent were used. The mobile phase flow rate was maintained at 2.5 mL min^{-1} throughout the run with a column temperature of 55 °C. The injection volume was 2 μL, and the detection wavelength was 280 nm.

2.3 Standard and sample preparations

100% methanol was used as a diluent for preparing the standards and samples. A standard solution was prepared by dissolving a specific amount of fulvestrant in the diluent, which was appropriately diluted to obtain a concentration of 1000 μg mL^{-1}. The sample solution of the API was prepared in a similar

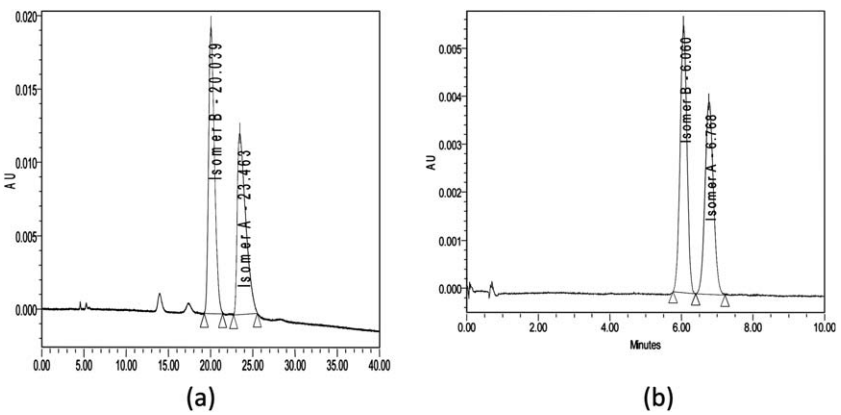


Fig. 2 Typical system suitability chromatograms (a) from the USP procedure and (b) from the SFC procedure.

Table 1 Forced degradation studies

	Fulvestrant isomer A			Fulvestrant isomer B		
Degradation conditions	Purity angle	Purity threshold	Purity flag	Purity angle	Purity threshold	Purity flag
Control conditions (no degradation)	—	—	—	—	—	—
Thermal degradation (at 60 °C for 72 h)	0.226	0.377	No	0.231	0.35	No
Photolytic degradation (UV)	0.279	0.472	No	0.268	0.418	No
Photolytic degradation (light)	0.217	0.365	No	0.216	0.329	No

way to the standard solution (1000 μg mL^{-1}). To prepare the injectable sample, a quantity of fulvestrant equivalent to 100 mg was placed in a 50 mL volumetric flask, 15 mL of the diluent were added, the mixture was sonicated for 5 minutes to dissolve the contents, and the flask filled up to the mark with the diluent to obtain a final concentration of 1000 μg mL^{-1}.

2.4 Forced degradation study

Forced degradation studies were conducted on the samples and on a plain placebo to prove the specificity of the method. Specificity measurements were carried out by exposing test solutions to heat (60 °C for 75 hours). Photolytic studies were carried out as per the current ICH requirements. The percentage and peak purity of fulvestrant diastereomer peaks was checked by using a PDA detector. The purity angle was within the purity threshold limit for the two peaks and demonstrated the analyte peak homogeneity. The results of the forced degradation studies are presented in Table 1.

2.5 Solution stability and mobile phase stability

The solution stability of the standard and the sample solution were assessed by leaving both the test solutions of the sample and the standard at room temperature for 24 hours. The mobile phase stability was also tested by keeping the mobile phase at room temperature for 3 days and evaluating the system suitability.

3 Results and discussion

3.1 Method development and optimization

3.1.1 SFC 1st tier screening. Chiral separation of drug substances usually involves the screening of a given set of solvents on several columns packed with stationary phases. This process is often time consuming and is generally regarded as a bottleneck prior to the analysis. A way to speed up the screening procedure is to improve the method development capacity using the UPC^2 multicolumn manage Aux. The first screening step was performed by using a generic method with a runtime of 15 minutes in 100% CO_2 and no organic modifiers, on four different stationary phases: BEH silica, BEH 2-ethyl pyridine (2-EP), CSH flourophenyl and amylose tris stationary phases with sub-2 μm particles, specifically designed for UPC^2 instrumentation. A sample containing a mixture of two pairs of diastereomers was injected onto the 4 columns in sequence. This first screening did not result in any separation of the two peaks. An attempt to improve the method was made by introducing a small quantity of β-CD (beta cyclodextrin) in a hydrophobic organic co-solvent. The results were not satisfactory, as very little separation was achieved.

3.1.2 SFC 2nd tier screening. Since fulvestrant is very hydrophobic and CO_2 is non-polar, the 2nd screening was done by introducing organic modifiers in various combinations with CO_2 on a Chiralpak AD-H column with the dimensions 150 mm × 4.6 mm, and a particle size of 5 μm.

For example, different organic solvents such as acetone, acetonitrile, ethanol, and methanol in various proportions were evaluated. These methods were programmed as isocratic runs with the same run time, temperature and back pressure settings as those in the generic method. Trials with acetone and acetonitrile resulted in no peak elution within 10 minutes. Trials with ethanol and methanol in different proportions resulted in the separation of the two isomers. The results are shown in Table 2.

From the screening process and solubility data (see Table 3), methanol was found to be the preferred solvent. The best resolution was achieved by adding 5% of acetonitrile in methanol with 75% supercritical CO_2 as the mobile phase.

Table 2 Resolution obtained from various combinations of organic modifiers

S. no.	Organic modifier(s)	Mobile phase A (%) carbon dioxide	Mobile phase B (%) organic modifier	Resolution
1	Acetone	80	20	No elution of peaks within 10 minutes
2	Acetonitrile	70	30	No elution of peaks within 10 minutes
3	Ethanol	70	30	1.47
4		75	25	1.61
6	Methanol	90	10	No elution of peaks within 10 minutes
7		75	25	1.47
8	5% acetonitrile in methanol	75	25	1.66[a]

[a] Highest resolution.

Table 3 Solubility study of fulvestrant

Solvent	Sample weight [mg]	Solubility[a]
Methanol	997	Very soluble[b]
Methanol–water (80 : 20)	1000	Not soluble
Methanol–water (70 : 30)	994.5	Not soluble
Acetone	1000	Freely soluble
Acetone–water (70 : 30)	999	Not soluble

[a] Solubility definitions: ~1000 mg of compound soluble in 1 mL – very soluble; 9 mL – freely soluble; 20 mL – soluble; 80 mL – sparingly soluble. [b] Highest solubility.

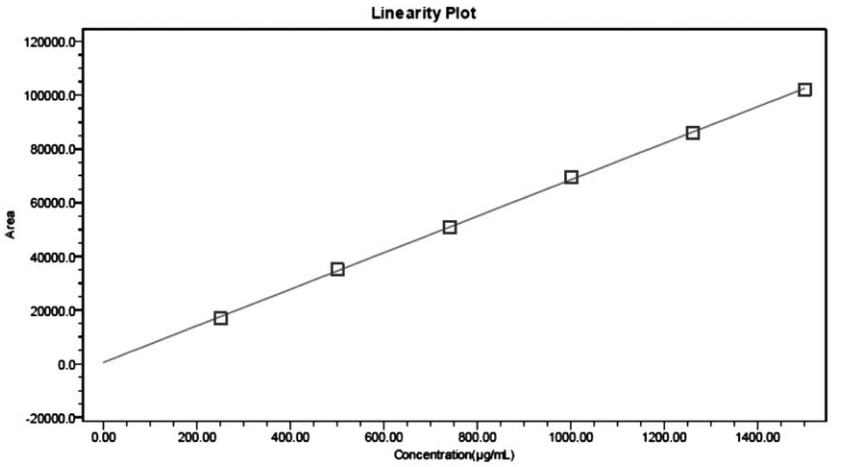
3.2 Method validation

The optimized method was validated as per the current ICH guidelines for the determination of fulvestrant diastereomers in the fulvestrant drug substance and formulations.[24]

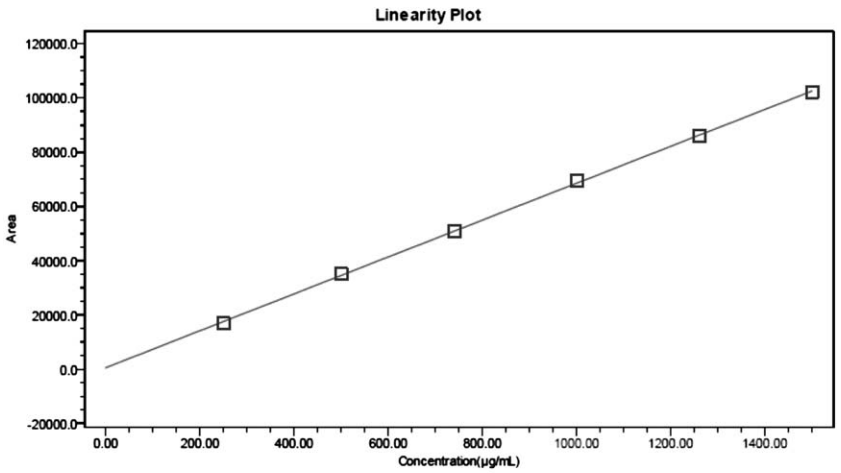
3.2.1 System suitability. The system suitability parameters were measured to verify the system performance. The system precision was determined in six replicate injections of standard preparations. The USP criterion for the resolution between the two diastereomers is not less than 1.3. In the current method a reproducible resolution of 1.6 was obtained.

3.2.2 Linearity. The linearity of the detector response was established by injecting the potential impurities at concentrations ranging from 25% to about 150% of the target concentration, and the correlation coefficient was determined to be 0.999. A linearity plot was made for the detector response dependent on the concentration, which is shown in Fig. 3.

3.2.3 Accuracy. The accuracy of the analytical procedure expresses the degree of closeness of the obtained results with the true values. The accuracy of the method was evaluated at three different concentrations, namely 2.5 μg mL^{-1}, 5 μg mL^{-1}, and 7.5 μg mL^{-1} of the drug product, and the recovery was



(a)



(b)

Fig. 3 (a) Linearity plot for isomer A and (b) linearity plot for isomer B.

Table 4 Regression, precision and accuracy

Parameter	Fulvestrant A	Fulvestrant B
Regression[a] equation (*y*)		
Slope	67.9628	77.5987
Intercept	552.4918	−19.9503
Correlation coefficient	0.9998	0.9999
Residual sum of squares	1 925 903.66	1 265 477.63
Precision (%RSD)[b]	0	0
Intermediate precision (%RSD)[b]	0	0
Accuracy	**% Recovery[c]**	
50%	101.4	99.4
100%	101.7	101.1
150%	100.2	100.6

[a] The linearity range is 25–150% with respect to 1000 μg mL^{-1} of fulvestrant. [b] Six determinations of each sample according to the method described at 1000 μg mL^{-1}. [c] %Mean recovery.

calculated for each added amount. The average recoveries and %RSDs were calculated. The recoveries ranged from 100.2% to 101.7%, and the %RSDs of the individual preparations were between 0.3% and 0.4%. The results of the accuracy studies are presented in Table 4.

3.2.4 Precision. The system precision was established by using the fulvestrant standard (100 μg mL^{-1}) in six replicate injections. The RSD(%) was calculated for the areas of the fulvestrant diastereomer peaks. The repeatability of the method was established by preparing and injecting six samples at 1000 μg mL^{-1}. The RSD(%) of the results obtained for the six samples was calculated. The intermediate precision was obtained by having the samples analyzed by a different analyst on another instrument and different day to evaluate the robustness of the method. The results of the precision studies are shown in Table 4. An overlaid chromatogram of the six sample solutions is shown in Fig. 4.

3.2.5 Robustness. The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate changes in the method parameters. To determine the robustness of the method, deliberate variations were made to the ABPR (active back pressure regulator), flow rate, column temperature and organic composition. The mobile phase flow rate was 2.5 mL min^{-1}; to study the effect of the flow rate on the resolution, it was altered to 2.25 and 2.75 mL min^{-1}. The impact of the column temperature on the resolution was studied at 50 °C and 60 °C. The variation in the organic composition in the mobile phase was also studied. In addition to the above parameters for UPC^2, a variation in the ABPR was also studied. This parameter helps to achieve the diffusivity of CO_2 to improve the resolution. No significant impact was observed on the resolution with changes to the ABPR. A minor reduction in the resolution was observed with increase in the column temperature and flow rate to 60 °C and 2.75 mL min^{-1}, respectively. The variation in the system suitability with respect to the robustness parameters is shown in Fig. 5.

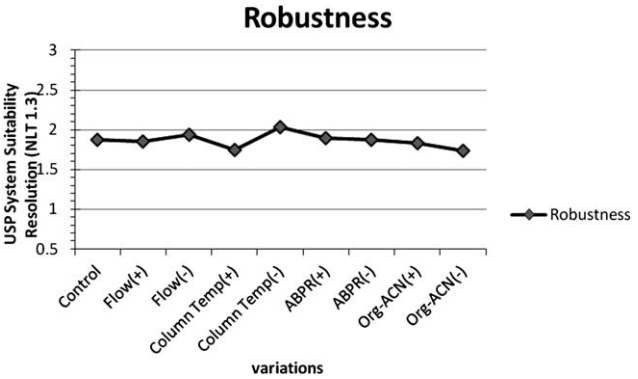


Fig. 5 Graph for the method robustness.

4 Concluding remarks

The rapid isocratic UPC^2 method developed for the quantitative determination of fulvestrant diastereomers is specific, precise, accurate, linear, and robust. The results obtained from the validation studies are satisfactory. This method exhibits excellent performance in terms of sensitivity and speed, and is also cost-effective. This technology is green and environmentally friendly in terms of low amounts of solvent waste generated. The method can be successfully employed for routine quality controls of high numbers of production batches in significantly less time.

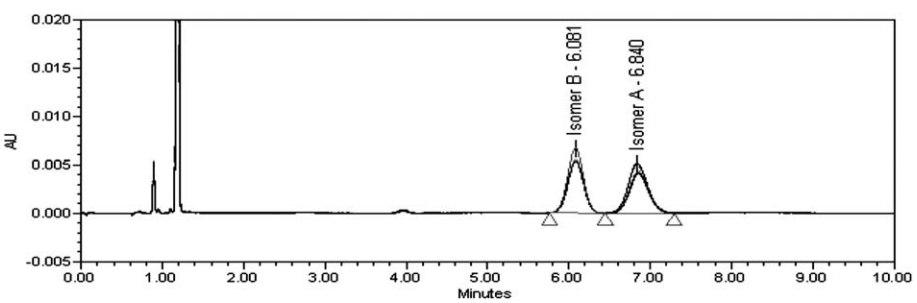


Fig. 4 Overlay chromatogram of six samples from the precision experiment.

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