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Supercritical fluid (CO_2) chromatography for quantitative determination of selected cancer therapeutic drugs in the presence of potential impurities

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In the present study, supercritical fluid (carbon dioxide) chromatographic methods were developed and validated for the quantitative assay determination of two cancer therapeutic substances, fulvestrant and azacitidine, using a UPC2 system. Fulvestrant was separated from six potential impurities, while impurities of azacitidine were separated using a 150 mm × 4.6 mm, I.D., a chiral column and 5 μm, particle size. Both drugs were analysed simultaneously in a single sequence using multiple channels in the system and respective methods. These new methods were validated for their intended purpose in accordance with the current ICH guidelines. The method exhibited excellent intra- and inter-day precision. A precision with RSD 1% and 1.6% was achieved for fulvestrant and azacitidine, respectively. A linear relationship r^2 > 0.999 was achieved between the concentration and detector response over a range of 25–150% of the target concentrations for both compounds. The two compounds were well quantified from their unspecified impurities obtained from stress studies. The method can be employed for routine quality control testing and stability analysis.

1. Introduction

Organic solvents are used as a medium to carry out reactions, extraction, separation, purification and drying in chemical reactions and in chemical analytical methodologies like spectrophotometry, chromatographic measurements and measurements of physicochemical properties. The majority of solvents are organic chemicals, some with hazardous and toxic properties. Most of them are costly and constitute part of the large waste by-products of the chemical industry, thereby causing environmental problems. Although most of their toxicities are known, prolonged and high concentration exposure can cause occupational diseases. Hence in this context, supercritical fluid CO_2 has emerged as a versatile solvent for various chemical separations such as supercritical fluid chromatography (SFC), an alternate and complementary method to HPLC.[1]

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 at a low-pressure drop without compromising the resolution, plate count and tailing. However, the lack of reliable and sensitive commercial SFC systems has prevented the extensive use of SFC in the industry.[3–5]

Azacitidine is 4-amino-1-β-D-ribofuranosyl-*s*-triazin-2(1*H*)-one with empirical formula $C_8H_{12}N_4O_5$. Its MW is 244. Azacitidine is a white to off-white solid. Azacitidine is insoluble in ethanol, acetone and methyl ethyl ketone; slightly soluble in ethanol/water (50/50), propylene glycol, and polyethylene glycol; sparingly soluble in water, water saturated octanol, 5% dextrose in water, *N*-methyl-2-pyrrolidone, normal saline and 5% Tween 80 in water; and soluble in dimethylsulfoxide (DMSO). The finished product is supplied in a sterile form for reconstitution as a suspension for subcutaneous injection or reconstitution as a solution with further dilution for intravenous infusion. Each vial of VIDAZA contains azacitidine and mannitol (each 100 mg) as a sterile lyophilized powder.[6] The structure of azacitidine is shown in Fig. 1a.

Fulvestrant is a novel endocrine therapeutic agent used for breast cancer with a unique structure and mode of action.

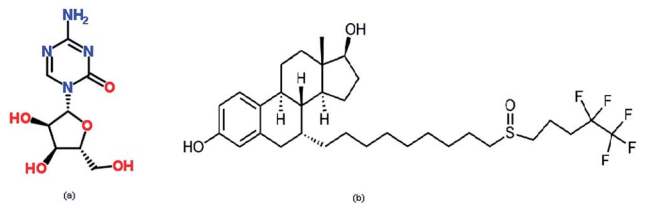

Fig. 1 Chemical structure of (a) azacitidine and (b) fulvestrant.

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Fulvestrant is the only parenteral agent in this category, which has well tolerated and low side effects. Fulvestrant is the subject of much ongoing research, with its novel mechanism and pharmacokinetic profiles to optimize its clinical efficacy.[7–10]

The chemical structure of fulvestrant is shown in Fig. 1b and 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 contains six asymmetric carbon atoms and a stereogenic sulphoxide in the side chain. The active ingredient is a mixture of two diastereoisomers: A and B, with the same absolute configuration at the stereogenic centers in the steroid system but with different absolute configurations at the sulphur atom. Each injection contains the following as inactive ingredients: alcohol, benzyl alcohol, benzyl benzoate, and castor oil.[11,12]

Currently, the fulvestrant drug substance is official in the United States Pharmacopoeia. Azacitidine for injection is available as a USP pending monograph.[13,14] Methods have been reported by the USP for fulvestrant and azacitidine drug substances.

The USP monograph for fulvestrant prescribes a single 70 minute HPLC gradient method for both assay and related substance determination. It utilizes a mobile phase consisting of solution A (water : acetonitrile : methanol 41 : 32 : 27) and solution B consisting of (acetonitrile : methanol : water 49 : 41 : 10). The method has a risk of high column back pressure, as it utilizes a shorter (150 mm × 4.6 mm I.D.) L7 column of smaller particle size (3.5 μm) with a high flow rate (2 mL min^{-1}). A survey of the literature shows that few analytical methods are available on the analysis of fulvestrant using high performance liquid chromatography or HPLC coupled with mass spectroscopy techniques.[15–22]

One validated high performance liquid chromatography method for the determination of fulvestrant in pharmaceutical dosage forms is reported by Varanasi, using a normal phase separation on a cyano column by employing a mixture of *n*-hexane and isopropyl alcohol as an eluent.[23] Cristian Fazioni[24] also has one US patent on the separation of fulvestrant isomers. The patent describes the methods for separating the isomers with a reverse phase HPLC using a chiral column using acetonitrile and hexane as mobile phase solvents.

Few methods are reported on azacitidine. One normal phase method was published on the estimation of an *n*-formyl impurity of azacitidine.[25] One HPLC method was published by Kissinger.[26] A patent has reported HPLC purity determination of 5-azacitidine.[27]

The challenges were then to develop methods for determination of these two compounds using low toxicity modifiers in the presence of their impurities.

2. Materials and methods

2.1 Chemicals and reagents

CO_2 was purchased from Sai Padmaja Oxygen at Hyderabad, India. Samples of methanol (HPLC grade) were obtained from Merck (India). Trifluoroacetic acid (TFA) was from Acros Organics Ltd Mumbai, India. Fulvestrant injection used in the study was prepared in the laboratory. Samples of the azacitidine drug substance were obtained internally from formulation development. Reference Standards of azacitidine and fulvestrant used are from USP.

2.2 Instrument and chromatographic conditions

An Acquity UPC^2 system from Waters Corporation (Milford, USA) equipped with a Waters photodiode array detector (PDA) was used in this study. The balance used for weighing the reference standards and samples was from Mettler and Sartorious. A single chiral column, Chiralpak AD-H-Diacel column (150 mm × 4.6 mm I.D.) with particle size 5 μm, was used for both the methods.

2.2.1 Chromatographic parameters for azacitidine. A mobile phase containing liquid CO_2 and methanol were pumped in a ratio of 65 : 35 into a chromatograph at a flow rate of 1.0 mL min^{-1}. The column was maintained at a temperature of 50 °C. A sample volume of 2 μL was injected into the chromatograph and detection was performed at 242 nm.

2.2.2 Chromatographic parameters for fulvestrant injection. Fulvestrant was quantified in the injection formulation using a mobile phase containing liquid CO_2 and a mixture of 0.25% TFA in methanol. A gradient programme [*T* (min)/%B: 0/40, 2.6/40, 3.0/50, 6.0/50, 6.2/40, and 8.0/40] was used to separate the analyte peaks. The chromatographic system was run at a flow rate of 2.0 mL min^{-1} and the column was maintained at a temperature of 50 °C. A sample volume of 1 μL was injected into the chromatograph and detection was performed at 225 nm.

Though the mobile phase, column temperature and flow rate are different, the two methods can be run simultaneously by creating two different method programmes.

2.3 Standard and sample preparations

Methanol was used as a diluent for preparing the fulvestrant standards and samples. DMSO (dimethylsulfoxide) was used as a diluent for preparing azacitidine standard and sample solutions. Fulvestrant standard and sample solutions were prepared by dissolving an amount of fulvestrant in the diluent and suitably diluted to obtain a concentration of 5000 μg mL^{-1}. For the preparation of the fulvestrant injection sample, a quantity of sample equivalent to 50 mg of fulvestrant was taken in a 10 mL volumetric flask, added to 4 mL of the diluent, sonicated for 2 min to dissolve the content and finally made up to the mark with the diluent to get the final concentration of 5000 μg mL^{-1}.

Azacitidine standard and sample solutions were prepared by dissolving an amount of azacitidine in the diluent and suitably diluted to obtain a concentration of 400 μg mL^{-1}.

2.4 Forced degradation study

Forced degradation studies were conducted on azacitidine APIs, samples of fulvestrant injection and placebo to prove the specificity of the method.

2.4.1 For azacitidine. Stress studies were carried out by exposing the samples to oxidation by hydrogen peroxide (10% H_2O_2, for 1 hour), acid hydrolysis (1N HCl for 30 minutes), base hydrolysis (1N NaOH for 45 minutes) and heat stress at 60 °C for

72 hours. Photolytic studies were carried out as per the current ICH guidelines.

2.4.2 For fulvestrant injection. Specificity studies were carried out by exposing the injection sample to oxidation by hydrogen peroxide (10% H_2O_2, for 1 hour), acid hydrolysis (1N HCl for 30 minutes), base hydrolysis (1N NaOH for 45 minutes) and heat stress at 60 °C for 72 hours. Photolytic studies were carried out as per the current ICH guidelines.[28] A mass balance was established between the unstressed sample and the exposed sample.

The homogeneity of fulvestrant and azacitidine peaks was established from the purity angle and peak threshold using a PDA detector (photodiode array).

3. Results and discussion

3.1 Method development and optimization

Azacitidine has three specified impurities namely Impurity A, Impurity B, and Impurity C. Azacitidine exhibits four chiral centers. There are six specified impurities in fulvestrant *i.e.*

Table 1 Results of precision and linearity from validation studies for fulvestrant and azacitidine

Fulvestrant					Azacitidine			
Tests	Method precision	Intermediate precision	Linearity		Method precision	Intermediate precision	Linearity	
1	95.1	96.8	Correlation	0.9996	98.1	99.2	Correlation	0.99996
2	97.1	96.6			101.9	98.9		
3	95.3	94.8			100.6	99.6		
4	94.5	96.6	Slope	303.1224	98.0	98.7	Slope	1260.3849
5	94.8	94.9			99.6	99.4		
6	95.3	95.9	Intercept	6965.8711	98.2	100.0	Intercept	1540.8662
Mean	95.4	95.9			99.4	99.3		
SD	0.91	0.89	Range (μg mL^{-1})	1239–7613	1.60	0.47	Range (μg mL^{-1})	3.695–615.866
%RSD	1	0.9			1.6	0.5		

Table 2 Results of accuracy from validation studies for fulvestrant and azacitidine

	Azacitidine			Fulvestrant		
	Level-1 (50%)	Level-2 (100%)	Level-3 (150%)	Level-1 (50%)	Level-2 (100%)	Level-3 (150%)
Sample-1	101.6	100.5	101.4	98	97.5	99.5
Sample-2	101.5	101.1	100.8	98	98.2	98.1
Sample-3	102.4	101.7	102.1	99.2	96.9	97.9
Mean recovery	101.8	101.1	101.4	98.4	97.5	98.5
%RSD	0.5	0.6	0.6	0.7	0.9	0.9

Table 3 Forced degradation parameters and results of assay and peak purity for fulvestrant and azacitidine

		Fulvestrant				Azacitidine			
S. no.	Stress condition	Assay	Purity angle	Purity threshold	Peak purity	Assay	Purity angle	Purity threshold	Peak[a] purity
1	Controlled sample (no degradation)	95.4	0.790	0.943	Pass	99.4	0.088	0.323	Pass
2	Spiked sample	95.5	0.903	1.262	Pass	98.8	0.118	0.343	Pass
3	Acid hydrolysis	91.8[b]	0.385	0.573	Pass	49.8[b]	0.406	0.474	Pass
4	Base hydrolysis	92.3	0.427	0.546	Pass	52.6[b]	0.622	1.046	Pass
5	Thermal (60 °C for 72 hours) degradation	93.4	0.601	0.980	Pass	98.3	0.089	0.333	Pass
6	Oxidation	91.4[b]	0.699	1.085	Pass	93.9	0.083	0.326	Pass
7	Photolytic degradation (UV) 200 watt	92.1	0.599	0.871	Pass	80.9[b]	0.080	0.342	Pass
8	Photolytic degradation (light) 1.2 million lux	93.2	0.696	0.877	Pass	96.7	0.183	0.323	Pass

[a] Peak purity passes if the purity angle is less than the purity threshold. [b] Maximum degradation observed.

6-keto-fulvestrant, D6, 7-fulvestrant, fulvestrant sulfone, fulvestrant extended, fulvestrant sterol dimer, and fulvestrant beta-isomer. Out of these, 'sulphone' is a potential degradant impurity. The method development was attempted and succeeded in two attempts for screening steps.

3.1.1 SFC primary screening. Waters approaches currently use four columns on waters, *i.e.*, UPC^2 (multi-columns manage Aux). These multi-column screening approaches allow the user to identify rapidly the right combination of mobile phase and column to achieve the desired separation.

The first screening step was performed by using a generic method with 15 minutes run time on four different stationary phases, BEH-silica, BEH-2-ethyl pyridine (2 EP), CSH-fluorophenyl and amylose tris, from waters, using 100% CO_2. The sample mixtures of fulvestrant injection and azacitidine with impurities were injected on each column in a sequence one after another. The first screening showed some separation on a silica column and chiralpak AD-H, which has given an insight into the nature of the impurities. In the second attempt, an organic modifier isopropyl alcohol at a 10% level was introduced and the sample matrices were injected. Separation was improved for azacitidine and its impurities, but no further improvement was seen for fulvestrant impurities.

3.1.2 Method optimization. Based on the above inferences, the "*chiralpak*" AD-H column was chosen for further analysis having dimensions 150 × 4.6 mm I.D. and 5 μm

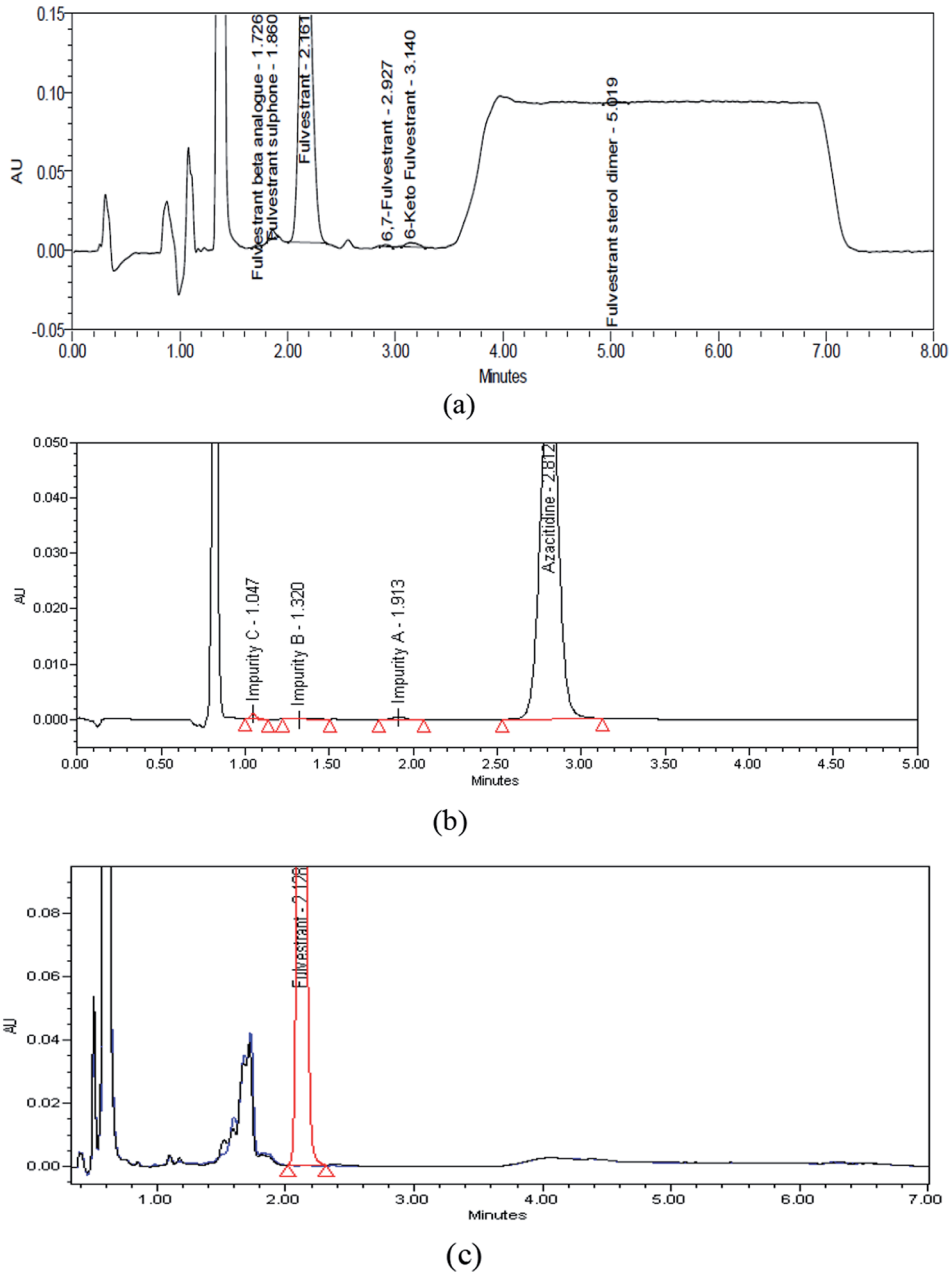

Fig. 2 (a) A chromatogram of the fulvestrant injection sample spiked with known impurities, (b) a chromatogram of the azacitidine sample spiked with its known impurities, and (c) an overlay chromatogram of the fulvestrant sample and placebo.

particle diameter. IPA was replaced with methanol in the same proportion to slightly increase the mobile phase polarity. A ratio of 75 : 25 (CO_2 : methanol), flow 2.0 mL min^{-1}, and ABPR (active back pressure) 1900 resulted in a better separation for fulvestrant and its impurities except for the sterol dimer. The fulvestrant sterol dimer exhibited a late eluting order, hence in order to reduce the run time, a simple linear gradient was introduced using methanol. A ratio of CO_2 and methanol of 65 : 35 resulted in an optimum separation for azacitidine from impurity A. This resulted in similar chromatographic parameters, which can be used simultaneously for both the compounds. After establishing the final chromatographic parameters, the stress samples were tested to confirm the stability indicating power of the methods.

3.2 Method validation

The optimized methods were validated as per the current ICH guidelines for validation of analytical procedures, *i.e.*, Q2 (R1).[26] The detailed validation experiments and results are discussed below.

3.2.1 System suitability. System suitability parameters were measured to verify the system performance. System precision was determined on six replicate injections of standard preparations and %RSD of six injections were evaluated and found to be below 2.0%.

3.2.2 Precision (repeatability). The precision of a method is the repeatability under the same operating conditions over a short interval of time. Repeatability is also termed as intra-assay precision.

The repeatability of the assay was determined by carrying out the analysis of the six samples. The relative standard deviation was calculated from the results of the obtained observations. These results are summarized in Table 1.

An RSD of 0.95% was found with a standard deviation of 0.9 for fulvestrant and an RSD 1.05% and 1.07 SD for azacitidine. This shows that the methods are precise as per the ICH and other guidelines.

3.2.3 Linearity. Linearity was established in a concentration range of 1239–7613 μg mL^{-1} (*i.e.*, 25–150% of target concentration) for fulvestrant. For azacitidine the linearity range was established between 3.695 and 615.866 μg mL^{-1}. The linear regression data from the calibration plot were indicative of an excellent linear relationship between the peak area and concentration over the range specified above and the data are provided in Table 1. A correlation greater than 0.999 was found for both fulvestrant and azacitidine.

3.2.4 Accuracy. The accuracy of the analytical procedure expresses the degree of the closeness of the obtained results with the theoretical values. The accuracy of the method was evaluated at three different concentrations namely, 50%, 100%, and 150% of the target assay concentration and % was calculated for each added amount. Table 2 summarizes the results of accuracy.

3.2.5 Specificity. Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradants, matrices, *etc.*

The specificity of the methods was determined by analyzing the diluent, the standard solutions, placebo and the samples spiked with individual known impurities. Samples of

Table 4 Robustness and method sensitivity data for method 1 for Fulvestrant

S. no.	Condition	RT of fulvestrant (Minutes)	USP resolution	USP tailing	%RSD of standard	Method sensitivity
1	Control (no change)	2.161	2.04	1.17	0.2	NA
2	Flow (+) 2.2 mL min^{-1}	1.954	2.13	1.09	0.5	Yes
3	Flow (−) 1.8 mL min^{-1}	2.409	1.6	1.19	0.7	Yes
4	Temperature (+) 60 °C	2.024	2.14	1.33	0.4	No
5	Temperature (−) (50 °C)	2.329	1.93	1.15	0.6	No
6	Active back pressure regulator (+) 2300	2.134	1.88	1.24	0.4	Yes
7	Active back pressure regulator (−) 2000	2.235	2.05	1.28	0.5	No

Table 5 Robustness and method sensitivity data for method 2 for azacitidine

S. no.	Condition	RT of azacitidine (minutes)	USP tailing	USP plate count	%RSD of standard	Method sensitivity
1	Control (no change)	2.813	1.0	3726	0.5	NA
2	Flow (+) 2.75 mL min^{-1}	2.678	1.0	4257	0.3	No
3	Flow (−) 2.25 mL min^{-1}	2.934	1.0	3167	0.4	No
4	Temperature (+) 55 °C	2.536	1.0	3466	0.2	Moderate
5	Temperature (−) (45 °C)	3.138	1.0	4059	0.5	Moderate
6	Active back pressure regulator (+) 2300	2.846	1.0	3712	0.4	No
7	Active back pressure regulator (−) 2100	2.768	1.0	3729	0.4	No

fulvestrant injection were subjected to stress conditions *i.e.* chemical conditions like acid hydrolysis, base hydrolysis, and oxidation, and physical conditions like treatment with heat, and light conditions (Table 3). Fig. 2a and b show specimen chromatograms of the samples spiked with known impurities. The impurities were well separated from the main peak indicating the specificity of the two methods. Fig. 2c shows an overlaid chromatogram of the fulvestrant injection sample and its placebo. No interference was observed at the retention of the fulvestrant peak.

The above data show that maximum degradation is observed in oxidation and acid hydrolysis with an assay value of 91.4% and 91.8% respectively in the case of fulvestrant injection. The data show that azacitidine is sensitive to hydrolysis under acids, bases, and light. The purity angle is less than the purity threshold under all stress conditions indicating that the principal peak is free from interference.

3.2.6 Robustness. The robustness of an analytical method can be measured by its capacity to remain unaffected by small but deliberate changes in the method parameters. The robustness of the method is determined by making deliberate variations in the flow rate, column temperature and ABPR (active back pressure regulator). The various altered conditions and the measured system suitability are shown in Tables 4 and 5.

4. Conclusions

The validation data concluded that the developed stability indicating assay methods were precise, accurate, linear and robust and found to be suitable for their intended purpose. The methods exhibit excellent performance and are superior over other existing methods in terms of sensitivity, speed, cost-effectiveness and ecofriendliness. The methods can be successfully employed for routine assay testing and release production batches for fulvestrant injection and azacitidine drug substances.

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