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Article

Rapid Liquid Chromatographic Determination of Itraconazole and its Production Impurities

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A simple, rapid ultra-performance liquid chromatography (UPLC) method was developed for the analysis of itraconazole and its associated production impurities. The optimum chromatographic conditions were achieved using an Agilent Zorbax Eclipse XDB C18 column, 1.8 μm (4.6 × 50 mm) installed in a column oven heater utilizing a gradient mobile phase of 0.08M tetrabutylammonium hydrogen sulfate buffer–acetonitrile (80:20, v/v), with ultraviolet detection at 235 nm. An Agilent 1200 RRLC Series was used for the UPLC analysis. UPLC is a technology that greatly reduces analysis time by utilizing columns packed with sub-2 μm particles. The method was validated according to International Conference on Harmonization guidelines with respect to precision, accuracy, linearity, robustness and limits of detection and quantification. All parameters were found to be well within the stated guidelines. The total analysis time was reduced by two–thirds, from over 30 min (the current European Pharmacopeia method) to under 10 min, and the method is applicable for assay and related substance determination. A method utilizing the sub-2 μm column on a conventional high-performance liquid chromatography system was also developed and validated, resulting in substantial time and solvent savings.

Introduction

Itraconazole, (+−)-*cis*-4-[4-[4-[4[[2-(2,4,-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylpropyl)-3H-1,2,4-triazole-3-one, is an oral triazole antifungal agent used for the treatment of infections caused by species of aspergillus, histoplasma and blastomyces. It acts by inhibiting the biosynthesis of ergosterol, a major component of the cell membrane of yeast and fungal cells. Itraconazole metabolizes to several metabolites, including a compound known as hydroxy-itraconazole, which has similar properties to the parent drug (1, 2).

The current standard method for the determination of itraconazole in active pharmaceutical ingredients (APIs) is in the European Pharmacopeia (3). Very little work has been conducted on the development and validation of a method for the examination of impurities in the bulk product. The object of this study is to develop a simple, fast and accurate method for the determination of itraconazole and its production impurities in the bulk drug product.

Much of the work in the literature has focused on the assessment of itraconazole and its metabolites. Gubbins *et al.* (1) developed a sensitive high-performance liquid chromatography (HPLC) method for the determination of itraconazole and its metabolite hydroxy-itraconazole in human serum. HPLC analysis was conducted on a Shimadzu system employing a C18 base-deactivated column packed with 5 μm particles (4.6 × 250mm). The column was heated to 37°C and the mobile phase was a 47:45:8 mixture of acetonitrile, 0.05M phosphate buffer and methanol. Consiglio *et al.* (2) developed another HPLC method for the therapeutic monitoring of itraconazole and its hydroxyl-metabolite in human serum. They developed their method on a Thermo HPLC system using a LiChrospher RP8 column packed with 5 μm particles (4.0 × 250 mm). A series of other works conducted in the area of method development for the HPLC assay of itraconozole and its hydroxy-metabolite have been reported in the literature (4–7).

Kumudhavalli (8) developed and validated a simple, specific, accurate and precise reversed-phase HPLC method for the determination of itraconazole in capsule dosage form. An Inertsil C18 5 μm column (250 × 4.6 mm) was employed in isocratic mode, coupled with a mobile phase consisting of a tetrabutylammonium hydrogen sulfate (TBAHS) buffer solution and acetonitrile in the ratio of 40:60 (v/v). The flow rate was 1.5 mL/min with detection at 225 nm. The retention time for itraconazole was 5.617min. The method was validated for linearity, accuracy, precision, specificity, limit of detection (LOD), limit of quantification (LOQ) and robustness. The LOD and LOQ were found to be 0.85 and 2.60 μg/mL, respectively, and recovery values of itraconazole from capsule formulation were found to be 98.3 to 103%. The system suitability parameters such as theoretical plates and tailing factor were found to be 2,927.43 and 1.08. The proposed method was successfully applied for the quantitative determination of itraconazole in capsule formulation (8).

The spectral characterization of itraconazole and two unknown impurities was conducted by Munigela *et al.* (9). The two impurities were detected in an itraconazole drug product by an isocratic reversed-phase HPLC method. The impurities were enriched by heat stress of the drug product and isolated for the degraded sample by column purification followed by isolation through preparative HPLC. Identification was based on the two-dimensional nuclear magnetic resonance spectroscopy and mass spectroscopy of the two impurities (9).

Method development and transfer

Whether analysts are developing methods for faster HPLC, or transferring from conventional HPLC to ultra-performance liquid chromatography (UPLC), several key points must be taken into consideration. When transferring from a column with a particular particle size to sub-2 μm particles and different column dimensions, the instrument setup must be considered to avoid band broadening. A successful method transfer requires the recalculation of the chromatographic parameters. An understanding of some practical calculations can help to achieve the correct scaling and maintain a consistent assay profile between the original method and the transferred method. The key areas to be considered when transferring to a shorter column with smaller particles are: flow rate, injection volume, analysis time and adjusted gradient profile (10).

There are scaling equations that can be used to calculate the adjustments that need to be made to conduct a successful transfer. Guillarme *et al.* (10) state that it is of prime importance to avoid any detrimental extra-column volumes when transferring to columns of small dimensions.

Therefore, if the method in question calls for a transfer to columns of different dimensions, then the following equation should be employed to modify the flow rate:

$$Flow_{col.1} \times (diameter_{col.2}/diameter_{col.1}) = Flow_{col.2} \quad (1.1)$$

Guillarme also advises to keep injection volume proportional to column to avoid extra-column band broadening by employing the following equation:

$$Inj.Vol._{col.1} \times (volume_{col.2}/volume_{col.1}) = Inj.Vol._{col.2} \quad (1.2)$$

The new analysis time may be calculated by the use of the following equation:

$$Time_{col.1} \times (length_{column2}/length_{column1}) = Time_{col.2} \quad (1.3)$$

These three equations are generally used in relation to isocratic elution; however, if the column dimensions or particle sizes of a gradient separation are going to be changed, then many other parameters have to be considered. For a shorter column, the analysis time is shorter; therefore, the gradient must be adapted proportionally. Instead of overall analysis time, it is now necessary to enter the timing and mobile phase composition for each segment of the gradient. Geometrical transfer of the gradient requires the calculation of the number of column volumes of mobile phase for each time interval (segment) of the gradient in the original method to ensure that the new calculated gradient takes place over the same number of column volumes. Other parameters that must be taken into consideration for very fast gradients are the dwell volumes of the old and new systems. To calculate the gradient profile, an equation can be derived to calculate each time segment of the gradient in the new method (t_{g2}), including column re-equilibration. The equation takes into consideration the volume of each column, the flow rate in the original method and the time segment of the original method. The equation is as follows:

$$t_{g2} = t_{g1} \times (V_{o2}/V_{o1}) \times (F_1 \times F_2) \quad (1.4)$$

where t_{g1} is original gradient (min), V_{o2} is new column void volume, V_{o1} is original column void volume, F_1 is original flow rate and F_2 is new flow rate.

The void volume of the column is the volume that is not occupied by the stationary phase, which is approximately 68% of the column volume.

Although the calculations of these equations are simple, they are tedious and time consuming. Most instrument manufacturers have software that performs the necessary scaling calculations to be conducted.

The primary difficulty in transferring gradient programs is related to dwell volume, because two instruments rarely exhibit the same dwell volume. Dwell volumes can range from a few microliters in modern instruments to a several milliliters in older instruments. Dwell volume refers to the system volume from the point at which the solvents are mixed until they reach the head of the column (11). At the start of the gradient program, the internal volume in the instrument is filled with the mobile phase that corresponds to the initial gradient conditions. As a result, the dwell volume of a system delays the arrival of the gradient at the column inlet. Therefore, the analytes, particularly the less retained ones, will isocratically migrate a certain distance through the column. Therefore, the gradient method will unavoidably have a two-step elution: an isocratic step at the beginning and then the gradient program. Although the primary effect of such an isocratic step will usually result in a retention time shift, the selectivity may also be affected. Therefore, when transferring gradient programs to instruments that have different dwell volumes, there may be different results on the same columns when running the exact same gradient program (12).

Dolan (11) suggests overcoming problems in transferring gradient methods to instruments with varying dwell volumes by adding the difference in delay time as an isocratic hold at the beginning of each run.

Another factor that must be taken into account when developing methods for faster chromatography, particularly when transferring compendia methods from columns of larger dimensions and larger particle sizes to smaller dimensions and particle sizes, are the limits imposed on any changes that can be made to avoid having to revalidate the method.

Majors *et al.* (13) discuss the problems that arise when a column is replaced with a new or equivalent column. They state that sometimes the chromatogram can be changed to the extent that it is no longer suitable for its intended use. They state that method adjustment is necessary in that case to correct the change. However, they also consider the extent to which the parameters can be changed before it becomes mandatory to revalidate the method. They state the following: method development refers to the initial selection of conditions for a proposed assay procedure. Method adjustment involves making minor changes in separation conditions for an already developed method, usually by means of correcting changes in separation as a result of a change in column. However, a major change in separation conditions is essentially equivalent to method development, as opposed to minor changes in method adjustments.

A company would have to carefully consider any changes to a method that exceed the maximum acceptable parameters outlined in the European Pharmacopeia. This would bring them into to the area of having to revalidate and re-lodge the method with the relevant regulatory authority. This is a costly and lengthy

process that would have to be economically justified. However, hundreds of method have been developed and transferred, leading to reduced analytical times, which is very beneficial in the pharmaceutical industry. Also, the transfer of conventional HPLC methods to faster and more efficient methods leads to the reduction of solvent use, which is economically beneficial in terms of solvent and solvent disposal costs. The reduction in solvent use is also more environmentally friendly.

Experimental

Materials and methods

Samples of itraconazole (CAS number 84625-61-6) and its production impurities (not commercially available) were received from Janssen Pharmaceutical (Cork, Ireland). The chemical structure of itraconazole and its production impurities are shown in Figure 1. HPLC-grade acetonitrile (ACN), methanol and TBAHS and analytical grade hydrochloric acid was purchased from VWR. Ultra-pure water was obtained using an EASYpure RO Reverse Osmosis Series 742 system.

Instrumentation

The following is the apparatus used for the first part of the development of the method. An Agilent HPLC 1100 series was used, equipped with an 1100 series binary pump (G1312A), an Agilent 1100 series autosampler (G1313A), an Agilent 1100 series diode array detector (DAD) (G1315A) for operation of

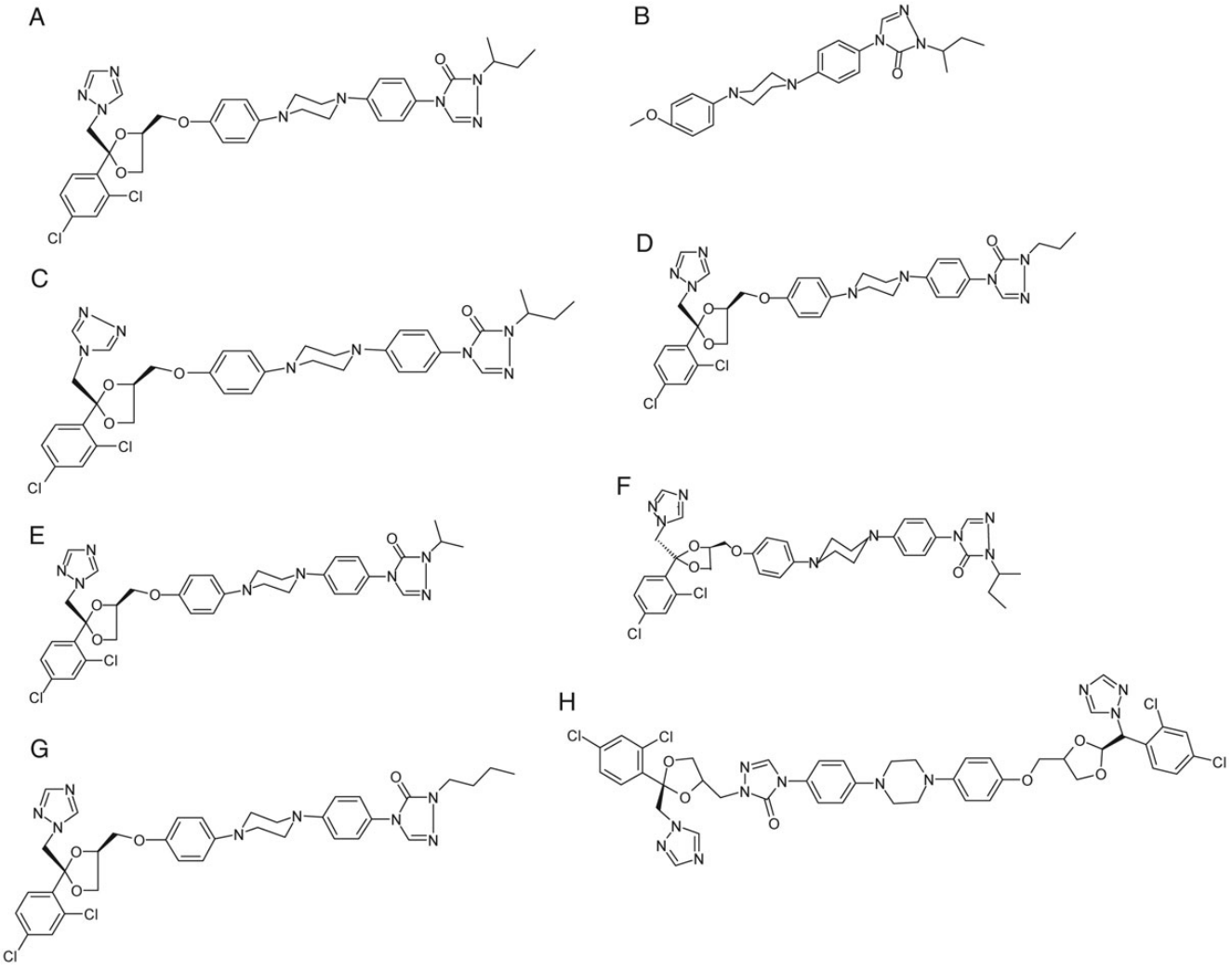

Figure 1. Structural formulae for itraconazole and its production impurities: Itraconazole, (+−)-*cis*-4-[4-[4-[[2-(2,4,-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylpropyl)-3H-1,2,4-triazole-3-one (A); Impurity A, (+−)-2,4-dihydro-4[4-[4-(-methoxyphenyl)-1-piperazinyl]phenyl]-2-(1-methylpropyl)-3H-1,2,4-triazol-3-one (B); Impurity B, (+−)-*cis*-4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(4H-1,2,4-triazol-4-ylmethyl)-1,3-dioxolan-4-yl] methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylpropyl)-3H-1,2,4-triazol-3-one (C); Impurity C, (+−)-*cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-y;methyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-propyl-3H-1,2,4-triazol-3-one (D); Impurity D, (+−)-*cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-l] methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylethyl)-3H-1,2,4-triazol-3-one (E); Impurity E, (+−)-*trans*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-2,4-dihydro-2-(1-methylpropyl)-3H-1,2,4-triazol-3-one (F); Impurity F, (+−)-*cis*-2-butyl-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1piperazinyl]phenyl]-2,4-dihydro-3H-1,2,4-triazol-3-one (G); Impurity G, (+−)-*cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methyl]-2,4-dihydro-3H-1,2,4-triazol-3-one (H).

up to 60 Hz, equipped with a 10 mm flow cell, a VWR model 2003 standalone vacuum degasser and a Shimadzu CTO-6A5 standalone column oven. Data acquisition was provided by an Agilent Chemstation, Rev. A. 10.02. (175). A Grant GD100 water bath was employed to heat the mobile phase. The following columns were employed to develop the method for itraconazole: a Thermo Hypersil BDS endcapped C18 column, 3.0 μm (4.6 × 100 mm), pore size 130Å; a Thermo Hypersil Gold C18 column, 1.9 μm (4.6 × 50 mm), pore size 175Å; an Agilent Zorbax Eclipse XDB C18 double endcapped base deactivated column, 1.8 μm (4.6 × 50 mm), pore size 80Å.

The following apparatus was used for the second part of the development of the method: an Agilent 1200 rapid resolution liquid chromatograph (RRLC) equipped with an Agilent 1200 series binary pump SL and vacuum degasser, an Agilent 1200 series high-performance autosampler, an Agilent 1200 series thermostatted SL column compartment and an Agilent 1200 DAD SL for up to 80 Hz operation, equipped with a 10 mm flow cell. Data acquisition was by Agilent Chemstation software, version B.02.01.SR1.

A Grant GD 100 water bath was employed to heat the mobile phase. An Agilent Zorbax Eclipse XDB C18 double endcapped base deactivated column, 1.8 μm (4.6 × 50 mm) was used to develop and validate the method for itraconazole.

Preparation of test solutions

A methanolic solution was prepared as the diluent for the standards. It was prepared by adding 4.0 mL of concentrated HCl to 500 mL of methanol. The solution was allowed to mix thoroughly and was completed to the mark in a 1.0 L volumetric flask. A 200 mg/L multicomponent stock solution of the seven impurities and the primary compound, itraconazole, was prepared by weighing 5 mg of each of the reference standards and adding to 10 mL of the methanolic solution in a 50 mL beaker. The reference materials were allowed to dissolve and completed to the mark in a 25 mL volumetric flask. A 100 mg/L standard solution of the combined seven impurities and the primary compound was prepared to develop the method and to conduct selectivity and robustness tests. It was prepared by placing 5.0 mL of the stock solution in a 10 mL volumetric flask and diluting to the mark with the methanolic solution. A series of standards in the following range: 5, 10, 20, 40, 80 and 120 mg/L were prepared from the 200 mg/L stock solution, for the purpose of conducting linearity tests.

A series of standards ranging between 10 and 50 mg/L were prepared to conduct injection and analysis precision. A series of standards in the range of 10, 50 and 120 mg/L were prepared to conduct intermediate precision. Solutions were stored in a cabinet at room temperature and were discarded after a period of 24 h.

Chromatographic conditions

The chromatographic conditions for the original method with the Hypersil BDS 3 μm column on Agilent HPLC 1100 series were as follows. The mobile phase was prepared by dissolving 27.2 g of TBAHS in ultra-pure water and completing to the mark in a 1.0 L volumetric flask, giving a concentration of 0.08M. The mobile phase was then filtered through a 0.45 μm nylon membrane filter and placed in reservoir A. The ACN was placed in mobile phase reservoir B. The mobile phase reservoirs were placed in a heated water bath, which was set at 30°C to heat the solvent. The column oven heater was set to 30°C.

The Thermo Hypersil BDS C18, 3 μm column (4.6 × 100 mm) was installed in the column oven heater. The column was conditioned by flushing with 100% ACN for 30 min and then with an 80:20 mixture of ACN and TBAHS for 5 min at a flow rate of 1.5 mL /min; the back-pressure at this flow rate was approximately 220 bar, but increased as the column aged. The injection volume was set to 10 μL with detection set to 225 nm. The following gradient was employed: 0–2.0 min, 80–20% (B); 2.0–22.0 min, 20–50% (B); 22.0–27.0 min, 50–50% (B); 27.0–28.0 min, 50–20% (B); 28.0–33.0 min, 20–20% (B).

The chromatographic conditions for the method utilizing the Zorbax XDB 1.8 μm and the Thermo Hypersil Gold C18 columns on the Agilent HPLC 1100 series were as follows. The mobile phase was prepared by dissolving 27.2 g of TBAHS in ultra-pure water and completing to the mark in a 1.0 L volumetric flask. The mobile phase was filtered through a 0.45 μm nylon membrane filter. The mobile phase reservoirs were placed in a heated water bath, which was set at 30°C to heat the solvent. The column oven heater was also set to 30°C. The column was installed in the column oven heater. The columns were conditioned by flushing with 100% ACN for 30 min and then with an 80:20 mixture of ACN and (0.08M) TBAHS for 5 min at a flow rate of 1.5 mL /min; the back-pressure at this flow rate with the sub-2 μm columns was approximately 270 bar and reached to greater than 330 bar as the columns aged. The injection volume was set to 5.0 μL with detection set to 225 nm. The following gradient was employed: 0–1.0 min, 80–20% (B); 1.0–11.0 min, 20–50% (B); 11.0–13.50 min, 50–50% (B); 13.50–14.00 min, 50–20% (B); 14.00–16.50 min, 20–20% (B).

The Agilent Zorbax Eclipse 1.8 μm column on the Agilent RRLC 1200 Series was conditioned in the same manner as detailed previously. The flow rate was optimized at 2.5 mL /min, with the optimized gradient as follows: 0–0.60 min, 80–20% (B); 0.60–6.60 min, 20–50% (B); 6.60–8.10 min, 50–50% (B); 8.10–8.40 min, 50–20% (B); 8.40–10.00 min, 20–20% (B).

Results and Discussion

Initially, itraconazole and its related impurities were analyzed by utilizing the original European Pharmacopeia method developed by Janssen Pharmaceuticals. The original method employed a Thermo Hypersil BDS C18 column (4.6 × 100 mm) packed with 3 μm particles. The total gradient run took a total of 33 min: 27 min for analysis, with the remaining 6 min for the re-equilibration of the column at the initial mobile phase composition of 80% A to 20% B (0.08M TBAHS–ACN). The resulting chromatograph is shown in Figure 2.

Development of the method

A rigorous examination of available columns that might be suitable for this analysis was conducted. The first column selected for the method development was a Thermo Hypersil Gold C18 (4.6 × 50 mm), 1.9 μm. This column was selected because it met with the same United States Pharmacopeia (USP) L1

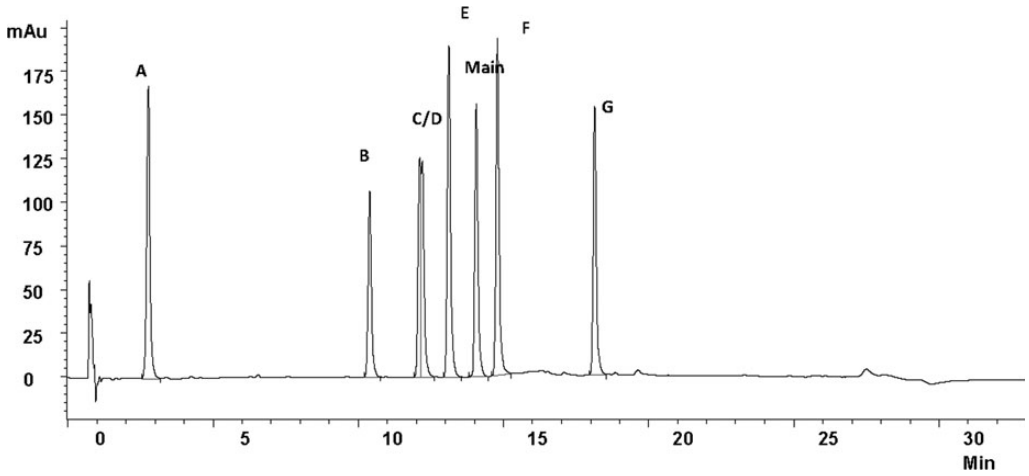

Figure 2. Chromatograph for itraconazole and its impurities utilizing the original gradient method.

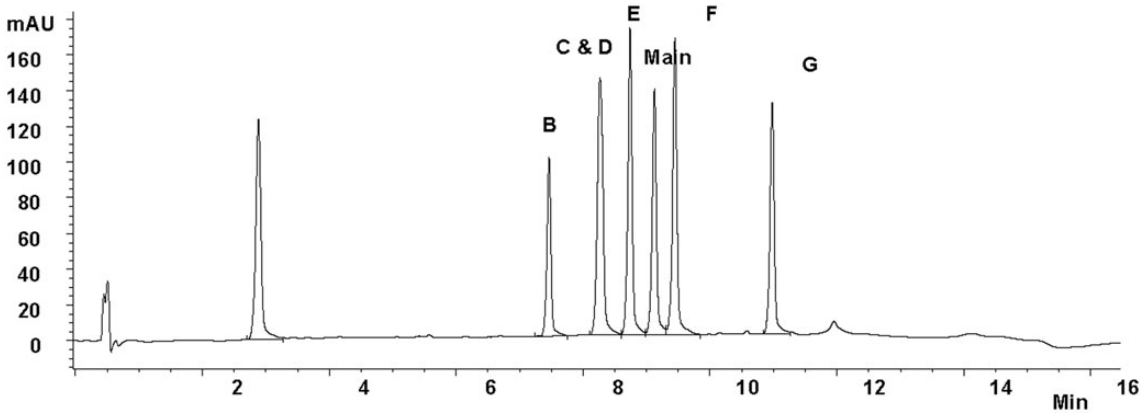

Figure 3. Chromatogram of itraconazole and its related impurities employing the Hypersil Gold 1.9 μm column.

classification as the original column. The pore size was slightly larger and the grade B silica was of higher purity. The gradient slope of the original method was input into the Agilent method translator (software that conducts the necessary scaling calculations), as were the column geometries, resulting in the following gradient: 0–1.0 min, 80–20% (B); 1.0–11.0 min, 20–50% (B); 11.0–13.50 min, 50–50% (B); 13.50–14.00 min, 50–20% (B); 14.00–16.50 min, 20–20% (B).

The method was further optimized by reducing the gradient run time, because it was felt that the resolution between the critical pair, the primary compound and Compound F, was more than sufficient at $Rs = 3.18$ with the first attempted gradient transfer. The optimized gradient method was as follows: 0–1.00 min, 80–20% (B); 1.0–8.0 min, 20–50% (B); 8.0–9.0 min, 50–50% (B); 9.0–9.50 min, 50–20% (B); 9.50–12.50 min, 20–20% (B). The new run time of 12.5 min allowed 9.5 min for the analysis time and 3.0 min for column equilibration at the initial conditions. The resolution of the compounds remained well within the specifications of the ICH guidelines, particularly for the critical pair.

With the new analysis conditions for the method established, a series of injections of multicomponent standards of the primary compound and the impurities were conducted to ensure that all peaks in the lower concentration levels could be detected. Multiple injections of the standards were also run individually to ensure that the order of elution had not changed from the original method. The results demonstrated that the newly developed method could detect all peaks in each concentration ranging from 5 to 120 mg/L and that the elution order had not changed. However, a major concern was the pronounced peak tailing that could be observed in the chromatogram for all compounds. The original method was developed on a BDS column, the silica of which has undergone a base deactivation process that results in reduced peak tailing for basic analytes. At first, it was thought that column aging was responsible for the peak tailing, but a new Hypersil Gold 1.9 μm column exhibited chromatograms with the same pronounced peak tailing (Figure 3).

Further research was conducted to source a suitable base-deactivated sub-2 μm column. The Agilent Zorbax Eclipse XDB C18 column (4.6 × 50 mm), packed with 1.8 μm particles, was considered as a substitute. The manufacturers claim that the extra dense bonding of the silica, which is then endcapped twice, produces a highly deactivated stationary phase that virtually eliminates peak tailing. The resulting chromatogram was a vast improvement on the Hypersil Gold column, with very

sharp peaks for all the compounds. Peak tailing was greatly reduced with excellent peak symmetries for each of the compounds. The retention times and resolution of the peaks were similar to those obtained with the Hypersil Gold column and the chromatogram obtained with the Zorbax Eclipse XDB C18 column is shown in Figure 4.

The method was transferred to the Agilent RRLC 1200 Series, on which higher pressures could be obtained. Various flow rates were tested, taking back-pressure and resolution into account. The optimum flow rate was determined to be 2.5 mL/min. At this flow rate, peak symmetry was excellent and the resolution between the critical pair of itraconazole and the F impurity remained satisfactory.

Method validation

The method was validated according to the guidelines laid down by the ICH (14), for the following parameters: linearity, accuracy, precision, selectivity, LOD, LOQ and robustness.

Linearity

For linearity, a series of multicomponent standards of itraconazole and its related impurities were prepared in the following range: 5, 10, 20, 40, 60, 80 and 120 mg/L. Each standard was injected six times and the percent relative standard deviation (RSD) values for the peak areas were determined. Calibration curves were constructed for itraconazole and each of the impurities and the response for each was found to be linear over the tested concentration range. The values for the coefficient of determination and the regression equations are shown in Table I.

Accuracy

The accuracy values for the primary compound and its related impurities were determined from the percent recovery of the linearity samples. The values for accuracy are given in Table II. All values were found to be within the acceptance limit for this method.

Precision

Precision was conducted in three stages: injection, analysis and intermediate.

Injection precision and analysis precision were conducted with consecutive injections of a multicomponent standard of the primary compound and its impurities prepared at two concentration levels, 20 and 80 mg/L, giving a total of 12 injections for each parameter. The results for analysis and injection precision were found to be well within the specified range of RSD $\leq$ 2%, as laid down by the ICH guidelines; these are shown in Table III for analysis and injection precision.

Intermediate precision

This was conducted in two stages by two separate analysts on separate days. For the first stage, multicomponent standards of the primary compound and its related impurities were prepared by Analyst One. Three separate standards were prepared

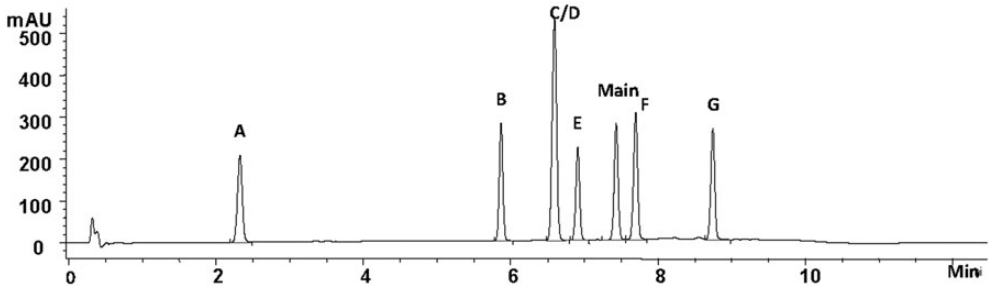

Figure 4. Chromatogram of itraconazole and its related impurities employing an Agilent Zorbax XDB 1.8 μm column on an Agilent 1100 Series, utilizing the optimised gradient method.

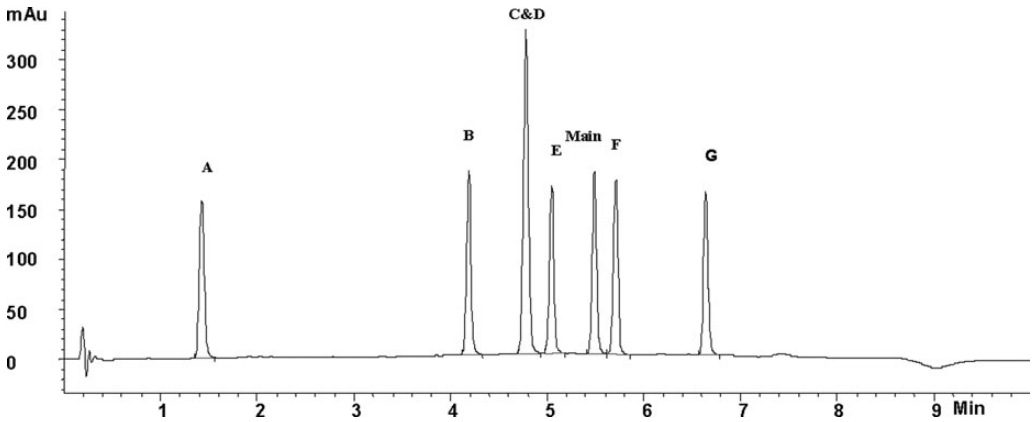

Figure 5. Chromatogram of itraconazole and its impurities developed on RRLC employing the Agilent Zorbax XDB 1.8 μm column under optimum conditions.

Table I
Linearity Regression Equations, Coefficient of Determination, LOD and LOQ Values for Itraconazole and its Related Impurities

Compound	Regression equation	r^2 values	LOD (mg/L)	LOQ (mg/L)
A	$y = 4.8691x - 0.3674$	0.9992	0.80	2.50
B	$y = 4.9885x + 4.5281$	0.9996	0.75	2.25
C/D	$y = 10.321x + 2.6317$	0.9997	0.68	2.28
E	$y = 4.6616x + 0.4446$	0.9995	0.65	1.50
Primary	$y = 5.1709x + 1.0623$	0.9992	0.50	1.80
F	$y = 4.8582x + 0.6637$	0.9994	0.60	1.85
G	$y = 4.7821x - 0.5263$	0.9997	0.70	2.10

Table II
Percentage RSD Values for the Accuracy of Itraconazole and its Related Impurities

Concentration	A	B	C and D	E	Primary	F	G
5 mg/L	99.60	101.28	100.20	102.46	99.62	101.50	101.25
10 mg/L	101.90	101.70	100.76	102.30	99.68	99.40	100.50
20 mg/L	102.10	102.60	102.40	101.24	101.70	101.09	100.70
40 mg/L	99.33	98.76	100.29	100.44	98.210	99.65	98.47
60 mg/L	102.40	100.80	100.40	98.510	101.70	100.40	98.69
80 mg/L	98.70	98.50	98.51	99.20	98.10	98.60	99.80
120 mg/L	102.60	98.10	99.70	101.20	99.45	100.90	99.77

Table III
Percentage RSD Values for Injection and Analysis Precision of Itraconazole and its Related Impurities

Injection precision			Analysis precision	
Compound	20 mg/L	80 mg/L	20 mg/L	80 mg/L
A	1.15	0.28	1.50	0.09
B	0.34	0.37	1.56	0.09
C/D	0.36	0.33	1.49	0.76
E	0.55	0.17	1.69	0.05
Primary	0.60	0.19	1.49	0.11
F	0.61	0.18	1.56	0.90
G	0.73	0.21	1.50	0.09

at three concentration levels to encompass the entire range. The concentration levels were 10, 60 and 120 mg/L. A single injection of each standard was conducted, giving a total of nine injections. The entire process was also conducted by a second analyst on a separate day. The results for intermediate precision were found to be well within the range of RSD $\leq 2\%$, as laid down in the ICH guidelines; they are shown in Table IV.

Limit of detection and limit of quantitation

Signal-to-noise ratios were used to determine the LOD and LOQ of itraconazole and its impurities. A signal-to-noise ratio of 3 for LOD and 10 for LOQ were the responses used for the determination of each test. A 5 mg/L standard was diluted with the diluent (methanolic solution) until a satisfactory signal-to-noise ratio was achieved. Once this level was found, six consecutive injections were conducted. The results for LOD and LOQ are shown in Table I.

Robustness

The robustness of this method was tested with respect to the effects that any minor variation in temperature would have on the retention times and the resolution of the critical pair. The robustness test was conducted at 29 and 31°C (the temperature of the method is normally maintained at 30°C). To test the robustness of the method, a series of six injections of a 120 mg/L multicomponent standard of the primary compound and its impurities were injected six times at a temperature of 29°C and six times at a temperature of 31°C. For this test, the standard deviation and RSD values for the retention times of all the compounds were calculated and any difference was found to be well below the upper limit of 2%. The same test was conducted for the resolution of all compounds; again, the results were found to be well below the upper limit of 2%. Although a slight reduction in the retention times of the compounds was found at the operating temperature of 31°C, this did not have any impact on the resolution of the critical pair. The conclusion is that the method is robust and reliable with respect to any minor variation of temperature.

Table IV
Percentage RSD Values for Intermediate Precision of Itraconazole and its Related Impurities

Concentration	A	B	C and D	E	Primary	F	G
10 mg/L	0.60	0.62	0.59	0.43	0.64	0.45	0.47
60 mg/L	0.10	0.45	0.64	0.41	0.44	0.29	0.38
120 mg/L	0.42	0.22	0.44	0.21	0.27	0.19	0.33
10 mg/L	1.07	0.86	0.71	0.81	0.90	0.94	1.32
60 mg/L	0.89	0.37	0.15	0.57	0.17	0.55	0.22
120 mg/L	0.11	0.19	0.14	0.37	0.23	0.40	0.26

Conclusion

The developed HPLC and UPLC methods for the determination of itraconazole and its related production impurities were found to be linear, precise, accurate and robust. Significant financial savings due to time and solvent reduction can be obtained by employing these newly developed methods: the analysis time was reduced from the original time of 33 min to 10 min. The sub-2 μm column can be utilized on the conventional HPLC system, but the optimization of the method is limited by the pressure restrictions of this system. (The upper pressure limit for conventional HPLC systems is 400 bar, whereas the upper limit on the rapid resolution liquid chromatography (RRLC) system is 600 bar). However, significant reductions in analysis time can still be attained. Janssen Pharmaceuticals, the company that initially developed the method, employs a series of system suitability tests involving a series of 13 injections. With the original Pharmacopeia method, the total analysis time for these tests can take 429 min, using 643.5 mL of solvent. The same system suitability tests utilizing the method developed with a sub-2 μm column can be conducted in less than half that time, using less than half the amount of solvent. The overall conclusion is that there are significant gains to be made for this assay in terms of efficiency, analysis time and a major reduction in solvent consumption with the use of the sub-2 μm column technology, whether it is employing the conventional HPLC system or the RRLC system.

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