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Development and validation of a reversed phase liquid chromatographic method for analysis of griseofulvin and impurities

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ABSTRACT

A simple and robust reversed phase liquid chromatographic method was developed and validated for the quantitative determination of griseofulvin (GF) and its impurities in drug substances and drug products (tablets). Chromatographic separation was achieved on a Discovery C_{18} (250 mm × 4.6 mm, 5 μm) column kept at 30 °C. The mobile phase consisted of a gradient mixture of mobile phase A (water–0.1% formic acid pH 4.5, 80:20, v/v) and B (ACN–water–0.1% formic acid pH 4.5, 65:15:20, v/v/v) pumped at a flow rate of 1.0 mL/min. UV detection was performed at 290 nm. The method was validated for its robustness, sensitivity, precision, accuracy and linearity based on ICH guidelines. The robustness study was performed by means of an experimental design and multivariate analysis. Satisfactory results were obtained from the validation studies. The use of volatile mobile phases allowed for the identification of three main impurities present above the identification threshold using mass spectrometry (MS). The developed LC method has been applied for the assay and impurity determination of GF drug substances and tablets. The method could be very useful for the quality control of GF and its impurities in bulk and formulated dosage forms.

1. Introduction

Griseofulvin (GF) was the first orally available antimycotic agent which has been in use for over forty years [1]. The drug is still relevant due to its reasonable price, what is certainly advantageous in developing countries. GF is produced by *Penicillium griseofulvum* or obtained by other means [2]. Related substances of GF were identified from fermentation media and include dechlorogriseofulvin, dehydrogriseofulvin, dihydrogriseofulvin, tetrahydrogriseofulvin, griseofulvic acid and isogriseofulvin [3]. The first two can also be formed by degradation. The chemical structures of griseofulvin and its related impurities are presented in Fig. 1.

The analysis of GF has been focused mainly on its determination in biological fluids such as plasma, serum and urine. These methods include thin layer chromatography (TLC) [4–6], LC [7–11] and gas chromatography (GC) [6,12–14]. Recently, an electrospray ionization LC–MS/MS method was validated and adopted for the determination of GF in human plasma [15]. Characterization and determination of GF in some other sample matrices such as moulds, marine strains, food and feeds and bioavailability studies have also been reported [16–22].

In the United States Pharmacopeia, both the drug substance and the dosage forms are assayed by LC using a stationary phase with nitrile groups bonded to porous silica particles [23]. However, there is no specification for related substances. The European Pharmacopoeia [2] describes a GC method for the determination of dechlorogriseofulvin and dehydrogriseofulvin while the assay of the drug substance is performed by UV spectroscopy. The International Pharmacopoeia [24,25] specifies a UV method for assay of GF without any method for related substances.

In literature, only a few methods have been reported for the analysis of GF in the drug substance or dosage forms. Some of the methods are rather old like the one of Holbrook et al. [26] who described in 1963 a chromatographic method for the assay of GF in the presence of several related substances employing Celite and measuring the eluate fractions by UV. In 1980, Townley and Roden [27] published an LC method using a Zorbax CN column to determine the purity of GF bulk drug substances, to assay GF in powders, tablets, capsules and to separate GF from its metabolites. Thoma and Kübler [28] carried out photodegradation of antimycotic drugs, flucytosine and griseofulvin and noted that dechlorogriseofulvin was formed in aqueous solutions as well as in ethanolic and methanolic solutions. The authors mentioned that in spite of the photo-instability of GF, no pharmacopoeia recommends storage protected from light.

As no up-to-date LC method is available at this moment for the determination of GF and its impurities, the development and

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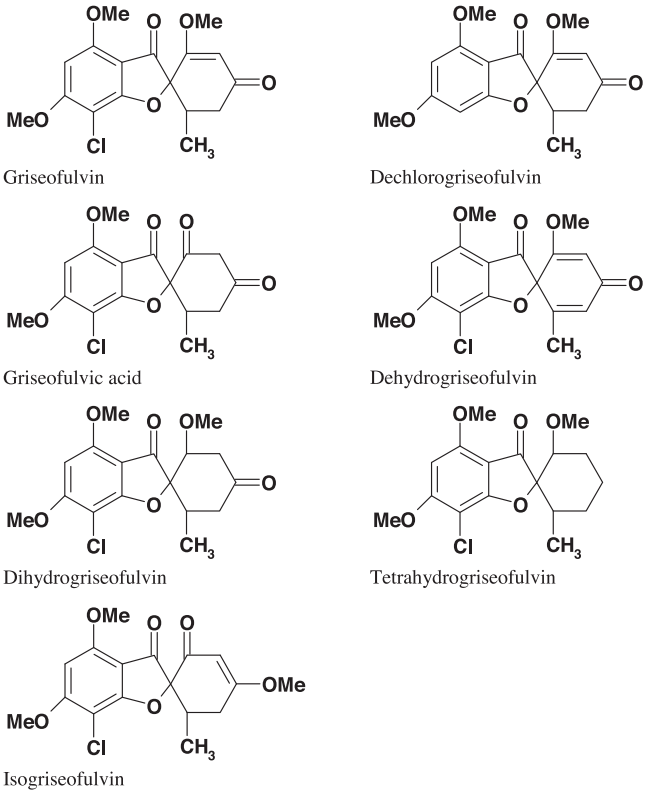

Fig. 1. Chemical structures of griseofulvin and its related impurities.

validation of such a method was the aim of this research. To be also applicable in developing countries, where GF is most administered, it was the intention to use conventional columns and equipment.

2. Experimental

2.1. Chemicals and reagents

HPLC gradient grade acetonitrile (ACN) was obtained from Fisher Scientific (Leicestershire, UK) and methanol (MeOH) from Acros Organics (Geel, Belgium). Stabilized tetrahydrofuran (THF), acetic acid and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Merck (Darmstadt, Germany). Phosphoric acid (H_3PO_4) was obtained from BDH (Briare, France) and formic acid (FA) from Biosolve Ltd. (Valkenswaard, The Netherlands). A Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to purify further demineralised water.

2.2. Samples and preparation of standard solutions

The drug substance utilized for method development was an old GF sample (15 years old) from LUDECO (Brussels, Belgium). This old sample was used in order to include as much as possible impurities from fermentation and degradation. For assay, GF drug substance obtained from SA Aca pharma NV (Waregem, Belgium) was used. Eight different commercial GF tablet samples were sourced from retail pharmacies in Ibadan, Nigeria. For investigation, a 0.5 mg/mL solution of GF was made in the mobile phase. For the assay of commercial tablets, a corresponding weight was taken. A 1.0% dilution was used as reference for the quantification of the related substances. Samples were filtered using a Chromafil® Xtra membrane filter, 0.2 μm obtained from Macherey-Nagel GmbH (Düren, Germany).

2.3. Instrumentation and liquid chromatographic conditions

2.3.1. Chromatography

The LC system from Dionex Softron GmbH (Germering, Germany) was equipped with a high pressure pump (P680ALPG), autosampler (ASI-100T) and UV/VIS detector (UVD170U). For data processing and acquisition, Chromeleon software version 6.80 from Dionex (Sunnyvale, CA, USA) was used. An ultrasonicator from Branson Ultrasonics Corporation (Danbury, CT, USA) and a pH metre from Metrohm (Herisau, Switzerland) were used to dissolve the sample and measure the pH of the mobile phase, respectively. The column was kept in a water bath at a temperature of 30 °C using a Julabo EM immersion thermostat (Seelbach, Germany). Final chromatographic separations were achieved on a Discovery C_{18} (250 mm × 4.6 mm, 5 μm) column obtained from SUPELCO (Bellefonte, PA, USA). The mobile phase was a gradient mixture of mobile phase A (water–0.1% formic acid pH 4.5, 80:20, v/v) and B (ACN–water–0.1% formic acid pH 4.5, 65:15:20, v/v/v) pumped at a flow rate of 1.0 mL/min. The gradient programme [time (min)/%B] was set as 0/50, 3/50 to 13/60 to 16/90, 20/90 to 24/50, 30/50. The gradient dwell volume of the system is 1.6 mL. The injection volume was 10 μL and UV detection was performed at 290 nm. The mobile phases were degassed by sparging with helium.

2.3.2. Mass spectrometry

An LCQ ion trap mass spectrometer from ThermoFinnigan (San Jose, CA, USA) equipped with an electrospray ionization source (ESI) interface operated in the positive ion mode was employed for the identification of impurities. The temperature of the heated capillary was set at 160 °C and the ion spray voltage was set at 4.50 kV. The capillary voltage was set at 10 V and the tube lens offset voltage at 5 V. Octapole 1 and octapole 2 offset voltages and the interoctapole lens voltages were set at −2.0 V, −7 V and −16 V, respectively. Nitrogen supplied by Air Liquide (Liège, Belgium) was used as sheath and auxiliary gas at a flow rate of 60 arb. u. and 20 arb. u., respectively. Helium was used as the damping and collision gas at a pressure of 0.1 Pa. For MS/MS investigation, the protonated molecule was isolated in the ion trap with an isolation width of 3 u and activated at a collision energy level (CEL) of 30%. LCQ Tune software (ThermoFinnigan) was used for instrument control, data acquisition and processing.

2.4. Robustness study

A robustness study was performed by means of an experimental design and multivariate analysis using Modde 5.0 software (Umetrics, Umeå, Sweden). Experimental factors that might potentially cause variability in the method responses were tested. Four factors (column temperature, pH of the mobile phase, amount of ACN and percent of formic acid) were investigated. A two-level full factorial design was applied with a number of runs equal to $2^k + n$, where k is the number of factors and n is the number of centre points. Thus, 19 experiments including 3 at the centre point were performed in triplicate. The lower and higher values for each factor in the design are given in Table 1. In every experiment, the percent of the 3 potential main impurities of GF (griseofulvic acid,

Table 1
Chromatographic parameter settings applied in the robustness investigation, corresponding to low (−), central (0) and high (+) levels.

Parameter	Low value (−)	Central value (0)	High value (+)
Temperature (°C)	28	30	32
pH	4.3	4.5	4.7
Acetonitrile in B (%, v/v)	64	65	66
% Formic acid	0.05	0.06	0.07