Journal of Pharmaceutical and Biomedical Analysis 121 (2016) 181–187

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Detection by LC–MS/MS of HIF stabilizer FG-4592 used as a new doping agent: Investigation on a positive case

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ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form 11 January 2016
Accepted 12 January 2016
Available online 15 January 2016

Keywords:
HIF stabilizer
EPO
Doping
LC–MS/MS
Athlete Biological Passport
FG-4592

ABSTRACT

Stabilizing the labile factor HIF (hypoxia inducible factor) for therapeutic use has led to the development of various molecules by pharmaceutical companies. These HIF stabilizers show promising erythropoiesis stimulating capacities and are of great interest for patients with chronical kidney disease and anemia. Amongst them FG-4592 from FibroGen is now under phase 3 of clinical studies. While this drug is still under investigation, a parallel market already allows to buy this product, which could be tempting for some athletes willing to increase their performances. To avoid such a use for doping purpose, WADA has listed HIF stabilizers and FG-4592 in particular as prohibited substances since 2011 and some anti-doping laboratories have developed a technique of identification of FG-4592 in urine. Here, we described the first case ever identified by an anti-doping laboratory of an athlete using FG-4592. Detection and confirmation in urinary samples was performed by LC–MS/MS. In addition, potential indirect markers erythropoietin (EPO) and hematological parameters followed in the Athlete Biological Passport (ABP) were analyzed during and after the period of use but showed no profound alterations. Only ABPS (abnormal blood profile score) reached (but did not exceed) the upper limit proposed by the ABP adaptive model just after the period of use of FG-4592. Altogether this case sends a warning for anti-doping laboratories which now must strengthen surveillance on HIF stabilizers and develop sensitive methods of detection for this new class of drugs.

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1. Introduction

Hypoxia-inducible factors (HIF) are transcriptional factors activated when oxygen availability decrease. In reaction, they induce the expression of a large panel of genes to limit the effects of hypoxia. This leads to the production of various proteins including the red blood cell activator erythropoietin (EPO), its receptor, glycolytic enzymes and the angiogenesis regulator VEGF [1,2]. In fact HIF factors are heterodimeric proteins composed of a beta subunit constitutively expressed and an alpha subunit, which is rapidly degraded by the proteasome under normoxia. This degradation is dependent of the activity of HIF prolyl hydroxylases (HIF-PH), which hydroxylate HIF alpha subunit on two proline residues thus prompting it to ubiquitination and degradation. However under hypoxia conditions, HIF-PH mediated hydroxylation of HIF alpha is inhibited. Consequently the HIF alpha subunit escapes from proteasome degradation, accumulates in the cytoplasm and then translocates in the nucleus where it dimerizes with HIF beta subunit to form the functional HIF transcription factor complex.

HIF-PH activity is dependent of the presence of ferrous iron, substrate 2-oxoglutarate and oxygen, the latter explaining its inactivation and subsequent HIF alpha stabilization when less oxygen is available. To block HIF-PH enzyme activity and stabilize HIF factors even under normoxic conditions, chemical analogues of 2 oxoglutarate have been developed opening the way for a therapeutic use [3]. Various pharmaceutical companies have developed HIF stabilizing drugs (like FG-2216 and FG-4592 by FibroGen, AKB-6548 by Akebia Therapeutics, GSK1278863 and GSK360A by GlaxoSmithKline, BAY85-3934 by Bayer). The most advanced in clinical assays are FibroGen drugs. They are derived from isoquinoline bound to a dipeptide that mimic 2-oxoglutarate and replace this co-substrate to block HIF-PH enzymatic activity with high efficiency (see Fig. 1

Abbreviations: AAF, adverse analytical finding; ABP, Athlete Biological Passport; ABPS, abnormal blood profile score; CKD, chronic kidney disease; EPO, erythropoietin; HGB, hemoglobin; HIF, hypoxia inducible factor; HIF prolyl hydroxylases, HIF-PH; IEF, isoelectric focusing; ISTD, internal standard solution; LC–MS/MS, liquid chromatography–tandem mass spectrometry method; LOD, limit of detection; PAAF, presumptive adverse analytical finding; RET, reticulocytes; WADA, World Anti-Doping Agency.

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http://dx.doi.org/10.1016/j.jpba.2016.01.029
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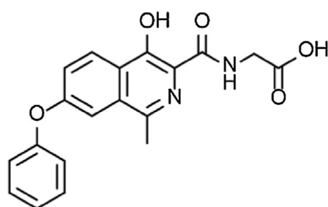

Fig. 1. Chemical structure of FG-4592 or roxadustat (mol $wt_{monoisotopique}$ = 352 Da).

for FG-4592 chemical structure) [4]. Pre-clinical studies in rodents have shown that FG-4592 (also called roxadustat) directly stimulates EPO and EPO receptor expression and enhanced maturation of erythroid progenitors. Repeated oral administration of this HIF-PH inhibitor led to increased red blood cell number, hemoglobin (HGB) and hematocrit at doses as low as 0.5 mg/kg with no adverse effects at doses as high as 200 mg/kg [5]. A randomized, single-blind, placebo-controlled, 4-week study of oral doses of FG-4592 (1–4 mg/kg) administered 2 or 3 times weekly (its half-life in circulation has been estimated to 11 h) in patients with chronic kidney disease (CKD) anemia showed that FG-4592 was well-tolerated and produced significant HGB increase in some subjects [6]. Available clinical data from phase 2 studies also showed that modest and intermittent increase in EPO induced by FG-4592 were sufficient to mediate erythropoiesis in non-dialysis patients with CKD, without increased incidence of hypertension or thrombosis [7,8]. FG-4592 also proved its efficacy in correcting anemia in incident dialysis patients regardless of baseline iron depletion: after 12 weeks of treatment HGB response (increase ≥1 g/dL from baseline) was achieved in 96% of patients while serum hepcidin levels were significantly reduced [9]. FG-4592 is now in phase 3 of clinical trial for the treatment of anemia in chronic kidney disease (CKD) patients with or without need of dialysis.

Each new erythropoiesis stimulator can be a potential doping substance used by athletes to increase their performances [10]. While still in development, molecules being studied in phase 2 clinical trials (or chemical copies) can nowadays easily be bought on parallel market. In particular substances labeled as FG-4592 are commercially available even if FibroGen has not yet officially disclosed the structure of the molecule. Well aware of this problem, the World Anti-Doping Agency (WADA) has listed HIF stabilizers as prohibited substances since 2011 [10]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a tool of choice for analysis of HIF stabilizers like FG-2216 and FG-4592 as described by Beuck et al. in 2011 [11]. However no case of doping with these drugs had been reported since then. Here, we describe the first case of doping with FG-4592 identified to our knowledge. In April 2015, direct identification of FG-4592 was established by LC–MS/MS in urine of an athlete. Various urine and plasma samples before and after the period of use were analyzed. The aims of this study were to present the sensitivity and large window of detection obtained with the analytical method used and to explore if FG-4592 treatment, with the protocol used by the athlete, affected the urinary and plasmatic EPO profiles and altered the hematological parameters measured as part of the Athlete Biological Passport.

2. Materials and methods

2.1. Chemicals and reagents

All solvents and reagents were of analytical grade purity. Acetonitrile and acetic acid were purchased from VWR chemicals (Radnor, PA, USA). Amonium formate was from Sigma (St. Louis, MO, USA). *Tert*-butyl methyl ether was from Biosolve (Valkenswaard, Netherlands). Ultra-pure water was produce using an ultra-pure water system (Milli-Q Millipore Corporation, Billerica, USA). The HIF stabilizing drug FG-4592 was obtained from Euromedex (Souffelweyersheim, France).

2.2. Screening by LC–MS/MS

Internal standard solution (ISTD) (50 μL, 50 ng/mL 17α-methyltestosterone) was added to 4 mL of urine followed by 1 mL of 1 M sodium phosphate buffer pH 6.5. Then, β-glucuronidase from *Escherichia coli* was added (50 μL) and hydrolysis was carried out 1 h at 55 °C. The buffered solution was alkalinized to pH 9 with carbonate bicarbonate solution and a solid phase extraction was then carried out with C18 cartridge and *tert*-butyl methyl ether as extraction solvent. The extracts were evaporated to dryness under nitrogen stream at 60 °C and the dry extract was reconstituted in 150 μL of the initial composition of the mobile phase and 20 μL were injected into the LC–MS/MS system.

LC–MS/MS analysis for the initial testing were carried out using triple quadrupole mass spectrometer (Xevo, Waters Associates, Milford, MA, USA) coupled to an ultra-performance liquid chromatographic (UPLC) system, (Acquity, Waters Associates) with positive electrospray ionization. The source parameters were as follow: gas flow desolvation: 800 L/h; gas flow cone: 50 L/h. Source and desolvation temperatures were set to 150 °C and 450 °C, respectively. Gradient elution was performed on an Agilent (Palo Alto, CA, USA) Zorbax SB-C8 column (2.1 × 100 mm, 1.8 μm particle size). The solvents used were: water containing 10 mM of ammonium formate and acetic acid (pH 4) (eluent A) and acetonitrile (eluent B). The gradient program started at 10% B and increased to 55% in 8 min, then increased to 100% in 0.1 min and decreased to starting conditions within 0.5 min. The column was re-equilibrated at 10% for 2 min. The flow rate was set at 400 μL/min and the column temperature was 20 °C. The samples were analyzed with a selected reaction monitoring (SRM) acquisition modes using diagnostic precursor/product ion pairs of *m*/*z* 353–278, *m*/*z* 353–250, and *m*/*z* 353–222. Collision-induced dissociation (CID) was conducted at optimized collision energies, and nitrogen was used as collision gas at 3.7×10^{-3} mBar.

2.3. Confirmation by LC–MS/MS

ISTD (50 μL, 4 ng/μL 17α-methyltestosterone) was added to 2 mL of urine followed by enzymatic hydrolysis as described in the initial testing sample preparation. The buffered solution was then adjusted to pH 4–5 with acetate buffer solution and extracted with 3 mL *tert*-butyl methyl ether. After shaking and centrifugation, the organic layer was evaporated to dryness under nitrogen stream at 60 °C. The residue was reconstituted into 150 μL of the initial composition of the mobile phase and 20 μL were injected into the LC–MS/MS system.

A Thermo Fisher Ultimate 3000HPLC system coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher, San José, CA, USA) was used for the confirmation procedure. The ion source was operated in positive electrospray ionization mode with the following MS conditions: Sheath gas (nitrogen): 50 psi; auxiliary gas (nitrogen): 10 psi; spray voltage 4.0 kV; heated capillary temperature: 300 °C; vaporizer temperature: 300 °C; collision gas (argon) pressure: 1.5 mTorr. The column used was a Zorbax XDB-C8 (2.1 × 150 mm, 5 μm particle size). The mobile phase consisted of A water containing 10 mM of ammonium formate and acetic acid (pH 4) and B acetonitrile and the gradient used was: 0–5.0 min 40–90% B, 5.0–9.5 min 90% B, 9.5–9.6 min 90–40% B, 9.6–15 min 40% B. The flow rate was set at 250 μL/min. The CID experiment was conducted at optimized collision energies with the

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