Journal of Pharmaceutical and Biomedical Analysis 148 (2018) 369–379

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Liquid chromatography-electrospray ionization-tandem mass spectrometry method for quantitative estimation of new imiqualine leads with potent anticancer activities in rat and mouse plasma. Application to a pharmacokinetic study in mice

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ARTICLE INFO

Article history:
Received 12 June 2017
Received in revised form
19 September 2017
Accepted 22 October 2017
Available online 24 October 2017

Keywords:
Imiqualines: EAPB02303 EAPB02302
Anticancer agents
LC/MRM-MS
Robustness
Rat mouse plasma
Pharmacokinetics

ABSTRACT

Imidazoquinoxaline derivatives (imiqualines) are a new series of anticancer compounds. Two lead compounds (EAPB0203 and EAPB0503) with remarkable *in vitro* and *in vivo* activity on melanoma and T-cell lymphomas have been previously identified. The modulation of the chemical structure of the most active compound, EAPB0503, has led to the synthesis of two compounds, EAPB02302 and EAPB02303, 7 and 40 times more active than EAPB0503 against A375 human melanoma cancer cell line, respectively. The aim of this study was to develop and validate a sensitive and accurate liquid chromatography-electrospray ionization-tandem mass spectrometry method to simultaneously quantify EAPB02303 and its potential active metabolite, EAPB02302, in rat and mouse plasma. Analytes were detected in multiple reaction monitoring acquisition mode using an electrospray ionization detector in positive ion mode. Following a liquid-liquid extraction with ethyl acetate, analytes and internal standard were separated by HPLC reversed-phase on a C18 RP18 Nucleoshell column (2.7 μm, 4.6 × 100 mm). The method was validated according to FDA and EMA Bioanalytical Method Validation guidelines. The robustness of the method was assessed by introducing small variations in nine nominal analytical parameters. Statistical interpretation was performed by mean of the Student's *t*-test. Standard curves were generated via unweighted quadratic regression of calibrators (EAPB02303: 1.95–1000 ng/mL, EAPB02302: 7.81–1000 ng/mL in rat plasma; EAPB02303: 0.98–1000 ng/mL, EAPB02302: 1.95–1000 ng/mL in mouse plasma). From the analysis of QC samples, intra- and inter-assay precision and accuracy studies demonstrated %R.S.Ds. <12.5% and percent deviation from nominal concentration <7%. Matrix effects (mean matrix factors from 91.8-108.5% in rat plasma; and from 90.4-102.4% in mouse plasma) and stability assays (recoveries >87%) were acceptable and in accordance with the guidelines. No quantifiable carryover effect was observed. The LLOQs were 1.95 ng/mL for EAPB02303 and 7.81 ng/mL for EAPB02302 in rat plasma, and 0.98 ng/mL and 1.95 ng/mL for the two compounds in mouse plasma, respectively. This method was successfully implemented to support a mouse pharmacokinetic study following a single intraperitoneal administration of EAPB02303 in male C57Bl/6 mice. The obtained pharmacokinetic parameters of EAPB02303 would be useful to optimize the dosing and the rhythm of administration for subsequent preclinical *in vivo* activity studies.

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1. Introduction

According to estimates from the International Agency for Research on Cancer (IARC), the cutaneous malignant melanoma incidence has presented an exponential increase around the world for over 7 decades [1,2]. Melanoma is an aggressive and heterogeneous skin cancer with a multifactorial development. The pathophysiological process implies a malignant transformation of the melanocytes under the influence of risk factors such as ultraviolet light exposure, genetic predisposition or also skin phenotype [3,4]. Despite the development of new anticancer agents by pharmaceutical industry [5], the mortality rate remains high and the

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https://doi.org/10.1016/j.jpba.2017.10.025
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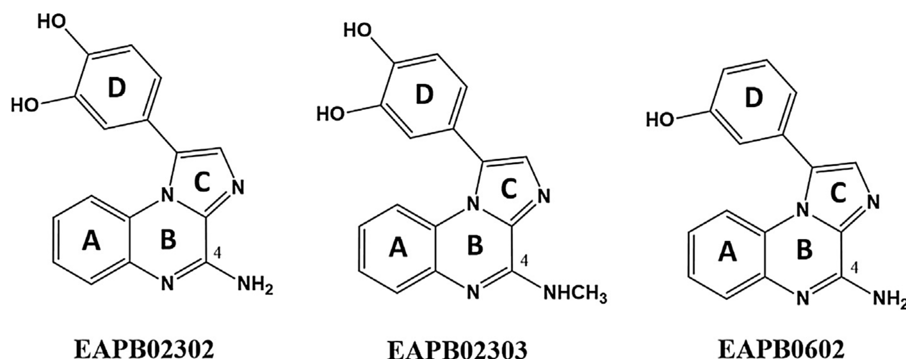

Fig. 1. Structural formulas of EAPB02302, EAPB02303 and EAPB0602 (internal standard).

cure rate low. Therefore, the development of compounds with original mechanisms of action is necessary.

Heterocyclic systems containing the quinoxaline moiety exhibit important biological activities. Many imidazoquinoxaline derivatives have shown interesting potential as active pharmaceutical products [6,7]. Our group is particularly involved in the study and development of new imidazo[1,2-*a*]quinoxaline compounds, also called imiqualines, which have been protected by different patents since 2008 [8] and granted in the USA on February 2013 and Canada on May 2016 [9,10].

Two active compounds, EAPB0203 and EAPB0503, have previously emerged as promising drugs with interesting antitumoral activities against a panel of human cancer cell lines including melanoma, T-lymphoma, myeloid leukemia and colon carcinoma [11–18]. EAPB0203 has shown significant *in vitro* cytotoxic activity against A375 human melanoma cell line, 110 and 45 times higher than fotemustine and imiquimod, respectively [11,15]. Tested *in vivo* in M4Be xenograft female Swiss Nude mice, EAPB0203 caused a significant decrease in tumor growth compared to vehicle control and fotemustine treatments [11,15]. EAPB0503 is 7–9 times more active than EAPB0203 on A375 human melanoma cell line and has been identified as our first lead compound [13,14]. Recently, EAPB0503 and its structural imidazo[1,2-*a*]quinoxaline derivatives have demonstrated major microtubule-interfering agents properties [19]. Studies to determine the absorption, distribution, metabolism, excretion, and toxicology properties of EAPB0203 and EAPB0503 have also been investigated [14,15,18,20,21].

The modulation of the chemical structures of the first identified lead compound (nature and/or position of the substituents) affects and modifies its biological properties. Among the synthesized compounds, EAPB02303 and its *N*-demethylated derivative, EAPB02302 (Fig. 1), have recently been identified as new lead compounds due to their *in vitro* activities [22].

To perform *in vivo* studies, a method for assaying these compounds in biological matrices should be developed and validated. Furthermore, pharmacokinetic data are required to optimize doses and rhythm of administration during preclinical activity studies.

In previous papers, we have found that, in all species, the main metabolite of EAPB0203 and EAPB0503 is formed after *N*-demethylation [14,15,18]. So, in the present paper, a liquid chromatography-electrospray ionization-tandem mass spectrometry method (LC-ESI–MS/MS) was developed and validated to simultaneous quantify EAPB02303 and its potential active metabolite, EAPB02302, in rat and mouse plasma. The robustness of the method was assessed by introducing small variations in nine nominal analytical parameters. Statistical interpretation was performed by mean of the Student's *t*-test. The choice of these two types of matrix is justified by the fact that during the preclinical development of drugs, rat is one of the principal species to be studied and mice xenografts are the most used *in vivo* melanoma models. Then, the validated method was used during a pharmacokinetic study performed in mice after intraperitoneal administration of EAPB02303.

2. Materials and methods

2.1. Chemicals and reagents

EAPB02303 (4-(4-(methylamino)imidazo[1,2-*a*]quinoxalin-1-yl)benzene-1,2-diol; molecular weight, 306 Da), EAPB02302 (4-(4-aminoimidazo[1,2-*a*]quinoxalin-1-yl)benzene-1,2-diol; molecular weight, 292 Da) and the internal standard (IS) EAPB0602 (3-(4-aminoimidazo[1,2-*a*]quinoxalin-1-yl)phenol; molecular weight, 276 Da) (Fig. 1), were synthesized by the "Oncopharmacochemistry and Cutaneous Pharmacotoxicology" laboratory (IBMM, Montpellier University, France). Briefly, they were synthesized in good yields via a bimolecular condensation of 2-imidazole carboxylic acid, followed by a coupling with ortho-fluoroaniline and subsequent substitution on the imidazole ring by Suzuki Cross-Coupling reaction using microwave assistance [22]. The purity of the compounds (higher than 99%) was evaluated by elemental analysis and NMR. They were stored at 20 °C protected from light.

Acetonitrile and ethyl acetate (HPLC grade) were obtained from Sigma-Aldrich (Saint-Louis, Missouri, USA). Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), isopropyl alcohol, and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Ammonium formate and formic acid were purchased from VWR Chemicals (Radnor, Pennsylvania, USA). All chemicals were of the highest purity available.

Six different batches of drug free rat (from healthy male Sprague Dawley rats weighting 230–280 g) and mouse (from Swiss mice weighting 20–25 g) plasma pools were obtained from Janvier Labs (Le Genest Saint Isle, France). These plasma pools were obtained from blood collected on lithium heparinate to prevent coagulation. Blank matrices were aliquoted and stored at −80 °C until used. The same lot was used during the study in the preparation of standards and quality control (QC) samples. The other lots were used to verify the specificity of the method and the absence of matrix effect. Formate buffer pH 3 was prepared by dissolving 126 mg of ammonium formate in 1 L of purified water; the pH was adjusted with formic acid. Purified water was generated by a Milli-Q reagent water system (Millipore, Bedford, MA, USA).

2.2. In vitro *cytotoxic activity study*

A375 cell line was obtained from American Type Culture Collection (Rockville, Md., USA). Cells were maintained in Gibco™ Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific, Illkirch-Graffenstaden, France) supplemented with 10%

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