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Quantification of EC-18, a synthetic monoacetyldiglyceride (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol), in rat and mouse plasma by liquid-chromatography/tandem mass spectrometry

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ABSTRACT

EC-18 (i.e., 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol), an active ingredient in Rockpid®, has been reported to be useful in controlling various types of inflammations, particularly those caused by neutropenia. Although this product was originally approved as a functional food in Korea, it is currently in phase II clinical trials for use in managing the severe chemotherapy-induced neutropenia in patients with advanced breast cancer who are receiving intermediate febrile neutropenia risk chemotherapy. The objective of this study was to develop a rapid, sensitive method for the determination of EC-18 in rat and mouse plasma and to evaluate the applicability of the assay in pharmacokinetic studies. EC-18 was extracted with MeOH from rat and mouse plasma samples, and the extract directly introduced onto an LC–MS/MS system. The analyte and EC-18-d3, an internal standard, were analyzed by multiple reaction monitoring (MRM) at m/z transitions of 635.4 → 355.4 for EC-18 and 638.4 → 338.4 for the internal standard, respectively. The lower limit of quantification (LLOQ) was determined at 50 ng/mL, with an acceptable linearity in the range from 50 to 10,000 ng/mL ($r > 0.999$) for both matrices. Validation parameters such as accuracy, precision, dilution, recovery, matrix effects and stability were found to be within the acceptance criteria of the assay validation guidelines, indicating that the assay is applicable for estimating EC-18 in concentrations in the range examined. EC-18 was readily determined in plasma samples for periods of up to 8 h following an intravenous bolus injection of 1 mg/kg in rats and at 5 mg/kg in mice, respectively, and up to 24 h following the oral administration of 2000 mg/kg in mice. The findings indicate that the current analytical method is applicable for pharmacokinetic studies of EC-18 in small animals.

1. Introduction

Cancer chemotherapy is often associated with a host of adverse effects, including neutropenia. This dose limiting toxicity typically causes a decrease in leukocyte and neutrophil counts resulting in the incidence of infections such as pneumonia and sepsis in patients to be significantly elevated [1,2]. As a standard therapy for this condition, granulocyte colony-stimulating factor is administered to patients receiving chemotherapy, although the treatment does not fully prevent the development of fevers and/or infections in immunocompromised subjects, especially elderly, patients [3,4].

1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (EC-18), a monoacetyldiglyceride, was originally isolated from antlers of the Sika deer (Cervus nippon Temminck) [5]. Although monoacetyldiglycerides are typically found in food, EC-18 can now be chemically synthesized in high yields by reacting glycerol, palmitic acid, and linoleic acid [6]. Rockpid®, a functional food product, contains EC-18 as the principal ingredient and was recently approved by Korean FDA. A literature search indicated that the compound was previously reported to stimulate the proliferation of hematopoietic stem cells and bone marrow stromal cells

Abbreviations: ACN, acetonitrile; AUC0→∞, area under the curve from time zero to infinity; AUMC0→∞, the area under the first moment curve from time zero to infinity; CL, clearance; Cmax, maximum concentration; FA, formic acid; IPA, isopropyl alcohol; IS, internal standard; MeOH, methanol; MRM, multiple reaction monitoring; MRT, the mean residence time; T1/2, half-life; Tmax, time point at maximum concentration; Vss, steady-state volume of distribution.

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in vitro/in vivo, and to inhibit hematogenous metastasis in of biliary cancer cells in a hamster model [5,7]. Furthermore, EC-18 may modulate eosinophil chemotaxis in epithelial cells, and effectively suppress neutrophilic inflammation [8,9]. In short, the compound is expected to be clinically useful for controlling/ameliorating conditions caused by inflammations. It was recently reported that the United States FDA approved phase II clinical trials of EC-18 for the management of severe chemotherapy-induced neutropenia in patients with advanced breast cancer who are receiving intermediate febrile neutropenia risk chemotherapy.

The absorption of glycerol and glycerides, probably EC-18 as well, proceed via complicated mechanism(s), as evidenced by their rapid decomposition during the digestion/absorption processes [10]. Furthermore, they may also be rapidly cleared by esterase enzymes that are produced in the body [11], suggesting that these compounds are likely to have a low bioavailability and short half-life. Therefore, protective formulation(s) that guarantee(s) the stability in the intestine and in the body may be necessary [12,13]. Despite the fact a number of studies regarding the pharmacological mechanism(s) of EC-18 have appeared in the literature, however, the pharmacokinetics and the mechanisms responsible for the absorption/elimination of EC-18 remains unknown. Therefore, the objective of this study was to develop and validate an analytical methodology according to guidelines set by the FDA [22] for the quantification of EC-18 in plasma samples obtained from small animal models. We were particularly interested in testing the applicability of the current method to pharmacokinetic studies involving small animals, since these animal models are likely to be useful in the early development of formulation for the compound. The findings indicate that the assay is capable of detecting EC-18 down to 50 ng/mL in rat and mouse plasma samples using only a limited sample size (50 μL), which will permit the method to be used in pharmacokinetic studies involving small animals.

2. Materials and methods

2.1. Chemicals and reagents

EC-18 (98.7% purity) and EC-18-d3 [an internal standard (IS)] were kindly provided by EnzyChem Lifesciences Co. (Seoul, Korea). Methanol (HPLC grade) and isopropyl alcohol (IPA, HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA). Formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO). Blank rat and mouse plasma samples containing heparin (an anticoagulant) were obtained from Orient Bio Inc. (Gyeonggi-do, Korea).

2.2. LC conditions

In this study, an HPLC system [Waters e2695 HPLC system (Milford, MA)], consisting of a binary pump, an online degasser, an autosampler, a column heater, and a reversed phase HPLC column [Synergi 4 μm Polar-RP 80 Å LC Column (150 mm × 2 mm, Phenomenex, Torrance, CA)] was used for chromatographic separations. The mobile phase A and B were composed of MeOH-IPA-FA (60:40:0.1, v/v/v) and 0.1% (v/v) FA in purified water, respectively. The flow rate of the mobile phase at 0.3 mL/min and the sample volume of 5 μL were used in this study. The analytical samples and column were maintained at 4° C and 30° C, respectively.

2.3. Mass spectrometer conditions

Mass spectrometric detection was performed with an API 3200 QTrap (Applied Biosystems, Foster City, CA), equipped with an electro-spray ionization (ESI) source operating in the positive-ion mode. In this study, the multiple reaction monitoring (MRM) method was used to detect the analytes: The MRM *m*/*z* transitions were monitored at 635.4 → 355.4 for EC-18 and 638.4 → 338.4 for the IS. The ion spray voltage, source temperature and pressure of the curtain gas were 5000 V, 200 °C and 10 psi, respectively. The declustering potentials for EC-18 and IS were 20 and 101 V, the entrance potentials were 10 and 6.5 V, respectively. The collision energies were 30 and 51 V, and collision cell exit potential were 6.0 and 4.0 V, respectively. In this study, data acquisition and processing were performed with the Analyst™ software (version 1.4.2; Applied Biosystems) running on a PC.

2.4. Standards and quality control (QC) samples

Stock solutions of EC-18 and IS were prepared in MeOH containing 0.1% (v/v) FA at concentrations of 1000 μg/mL and 1000 ng/mL, respectively. A batch of EC-18 standard solutions and QC solutions was prepared by the serial dilution of the stock solutions with MeOH containing 0.1% FA (v/v). A 5 μL aliquot of an EC-18 standard solution was added to 45 μL of blank rat or mouse plasma for the preparation of calibration standards containing EC-18 at 50, 100, 250, 500, 1000, 2500, 5000 or 10000 ng/mL. Using a similar dilution method, a batch of QC samples was prepared so as to have concentrations of EC-18 at 50, 150, 1000, and 8000 ng/mL in blank plasma. The samples were then processed similar to the procedure described in Section 2.5 (see below).

2.5. Sample preparation

A 50 μL aliquot of plasma sample was transferred to a SafeSeal Microcentrifuge Tube (Sorenson BioScience, Inc., Murray, UT). A 200 μL aliquot of IS solution (200 ng/mL in MeOH containing 0.1% FA, v/v) was added to the sample, followed by vortexing for 10 min. The mixture was then centrifuged at 16,100*g* at 4 °C for 10 min and the supernatant transferred to an analysis vial. A 5 μL aliquot was injected onto the LC–MS/MS system.

2.6. Method validation

2.6.1. Selectivity

In this study, six lots of plasma samples from six different rats/mice were prepared to evaluate the selectivity of the assay. In particular, the presence of any interfering peak in double blank samples (i.e., samples without EC-18 and without IS), blank samples (i.e., blank plasma added with the IS only) and the lower limit of quantification (LLOQ) for the samples was carefully monitored.

2.6.2. Linearity

Calibration curves, in the concentration range from 50 to 10,000 ng/mL, were constructed with the ratios of the peak area of EC-18 to that of the IS against the EC-18 concentration in the plasma standards. The calibration curves were fitted using a linear regression model with intercept and weighing factor of 1/standard concentration for the data.

2.6.3. Precision, accuracy and dilution

Three batches were prepared on different days to determine the precision and accuracy of the assay. Each batch consisted of six replicates of QC samples at concentrations of 150, 1000 and 8000 ng/mL and LLOQ samples (50 ng/mL). Another batch was prepared with six replicate samples containing 80,000 ng/mL and diluted tenfold with blank plasma to give the expected concentration at 8000 ng/mL. All samples were then processed according to the procedure described in the previous section.

The precision was determined as the percent coefficient of variation at each concentration. The accuracy was estimated by calculating the percent difference between the calculated and theoretical concentrations.