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Validated LC-MS/MS method for simultaneous analysis of 21 cephalosporins in zebrafish for a drug toxicity study

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ABSTRACT

Zebrafish model was used to perform this drug toxicity study. In this study, we monitored the absorption of drugs in zebrafish. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed in this experiment. The LC-MS/MS method was used for the simultaneous quantification of 21 cephalosporins in zebrafish. To prepare samples, rapid protein precipitation was performed with 1% formic acid in methanol. All the analytes were separated on a C_{18} column. These analytes were analyzed by performing multiple-reaction monitoring (MRM) scans in positive electrospray ionization mode. The ranges for limits of detection (LOD) and quantification (LOQ) were as follows: 0.01–10 ng/mL (S/N ≥ 3) and 0.05–25 ng/mL. Intra-and inter-day accuracy of all 21 cephalosporins were within the range of 80%–120%. Intra-and inter-day precision were within the range of 0.4%–11.2%. All the 21 analytes were analyzed within 10 min. In drug toxicity studies, the present method can be used to determine the internal concentrations of cephalosporins in zebrafish following the administration of drugs.

Introduction

In some fields of biomedical research, the zebrafish (*Danio rerio*) model is being used increasingly for drug screening, pharmacological safety studies, and toxicity assessment. This convenient animal model can be used to bridge the gap between performing cell-based assays and conventional animal testing. Although in vitro assays are usually performed on cultured cells to evaluate potential drug effects, they frequently fail to predict the complex metabolism that affects drug efficacy and causes toxicity in animals. The experimental advantages of this vertebrate model are as follows: i) drug can be delivered directly in the fish water; ii) a small amount of drug is required for each experiment; iii) each test is performed on a statistically significant number of animals; and iv) all the tests can be performed at a low cost. It is possible to visually assess compound-induced effects on the morphology of zebrafish because they have transparent bodies [1]. Zebrafish larvae share physiological, morphological, and histological similarities with mammals, so they are considered as valuable models for evaluating the toxicity and safety liabilities of drug candidates [2–13].

In most zebrafish assays used to analyze drug toxicity, the zebrafish is exposed to the drug in aqueous medium, and drug uptake takes place predominantly through diffusion through the skin [9,11,12]. The zebrafish larvae are then examined to determine morphological and behavioral phenotypes and lethal concentration (LC_{50}). Drug uptake is governed by the physicochemical properties of each compound. The aqueous concentration of the drug in the exposure medium does not necessarily reflect the internal concentration (body burden) in the embryo or larvae of the zebrafish [9]. The internal concentration should be determined to correlate any toxic phenotype with the actual concentration of the drug within the larvae of zebrafish. In a previous study, fertilized eggs were exposed to different concentrations of 15 compounds (eight compounds had teratogenic potential, while seven compounds had non-teratogenic potential) for 96 h post-fertilization. Then the level of compound uptake and 28 morphological endpoints were assessed in these fertilized eggs. By determining compound uptake, they confirmed whether each compound had teratogenic potential [6]. In a previous review, researchers have reported that liquid chromatography–mass spectrometry (LC-MS) technology can be used to correlate any toxic phenotypes with the body burden of a compound; moreover, potential false-negative and false-positive results can be identified with LC-MS technology. By determining the effective concentrations of compounds, researchers can help facilitate investigations that strive to translate zebrafish results into rodent and human toxicity data [14].

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In the past decade, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been extensively used to analyze small molecules in biological matrices [15–18]. This is because the LC-MS/MS technique has high specificity, selectivity, and sensitivity. This technique has also been used to determine internal concentrations of several types of compounds in zebrafish, such as pharmaceuticals, pesticides, and metabolites [19–25]. Several LC-MS/MS methods on determination of cephalosporins in pork muscle and milk have been published [26,27]. However, all the previous methods were in different matrices, the results could not be readily applied to zebrafish samples.

In previous studies, our group of researchers determined the toxic effects of cephalosporins by zebrafish embryo toxicity testing [28–32]. Our preliminary conclusion was as follows: the toxic effects of drugs were governed by both toxic functional groups and drug absorption. In this drug toxicity study, our main aim was to develop a rapid and sensitive LC-MS/MS method for the simultaneous quantification of 21 cephalosporins in zebrafish. The real concentrations of cephalosporins can be determined accurately with this fast, simple, and sensitive method. With this approach, the toxicity results can be correlated with drug body burden. Potential false-negative and false-positive results can then be identified. In this experiment, we developed an innovative method to determine effective concentrations; these concentrations could be useful in translating zebrafish results into rodent and human toxicity data.

Experimental

Chemicals and reagents

The reference standards, cefalexin (130408–201411, 94.4%), cefradine (130427–201107, 88.3%), cefadroxil (130431–201203, 94.8%), cefaclor (130481–201205, 95.3%), ceftizoxime (130504–200702,98.2%), cefdinir (130502–201403, 98.0%), cefuroxime (130493–201105, 92.1%), cefoxitin (130572–201102, 95.1%), ceftezole (130510–201402, 90.9%), cefazolin (0421–9603, 99.3%), cefotaxime (130483–201505,92.2%), cefathiamidine (130523–201203, 99.0%), cefepime (130524–200903, 83.5%), cefmenoxime (130525–201001, 94.6%), cefpirome (130545–201701, 83.0%), cefminox (130508–201003, 76.9%), cefotiam (130565–201703, 81.3%), ceftazidime (130484–201205, 85.7%), cefodizime (130520–200902, 89.0%), cefoperazone (130420–201105, 93.8%) and clenbuterol (100072–201503, 90.5%, internal standard (IS)) were purchased them from the National Institutes for Food and Drug Control (Beijing, China). Flomoxef (5005, 95.8%) was donated by SHIONOGI&CO.,LTD. (Fig. 1). Ammonium hydroxide and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was purchased from Quchenshi Company (Guangdong, China).

A stock solution (1.0 mg/mL) of each cephalosporin was prepared by dissolving each reference standard in deionized water; each stock solution was stored at −70 °C. A primary working solution (10 μg/mL) of each analyte was prepared by diluting each stock solution with a 5 mM ammonium formate–acetonitrile mixture (90:10, v/v). To prepare working solutions (3000, 1500, 600, 450, 300, 150, 60, 30, and 6 ng/mL) of 21 cephalosporins, we mixed working solutions of each analyte (10 μg/mL) and diluted the resultant solution with the 5 mM ammonium formate–acetonitrile mixture (90:10, v/v). The resultant solution was stored at 4 °C. A working solution of clenbuterol (IS, 200 ng/mL) was prepared by diluting the stock solution with 5 mM ammonium formate-acetonitrile mixture (90:10, v/v); the resultant solution was stored at 4 °C.

A stock solution of ammonium formate buffer was prepared from ammonium formate (1.0 M) and formic acid (1.0 M); this stock solution was stored at 4 °C. The unadjusted pH of the buffer was 3.5. To prepare formate buffer (5 mM) of lower concentration, the stock solution was diluted with deionized water.

Preparation of calibration samples

In this study, blank zebrafish homogenate was prepared from wild zebrafish (three days post-fertilization, 3 dpf). Thirty wild zebrafish were triturated with 100 μL deionized water. These wild zebrafish were then homogenized for 1 min. A 10-μL aliquot of each working standard solution was added to 50 μL blank zebrafish homogenate in a pre-labeled 1.5 mL microcentrifuge tube (Axygen, CA, USA). Then we prepared the following concentrations of calibration samples: 1, 5, 10, 25, 50, 75, 100, 250, and 500 ng/mL.

Sample preparation

Zebrafish homogenate (50 μL) and IS working solution (10 μL) were individually transferred into a 1.5-mL microcentrifuge tube. Then, we added 200 μL of 1% formic acid-methanol solution to the tube. The samples were vortexed for 20 s, and they were centrifuged at 10800 g for 10 min to precipitate protein. The resultant supernatant (10 μL) was injected into LC-MS/MS for analysis.

LC-MS/MS conditions

An LC-MS/MS system consisted of following components: a 20A LC instrument (Shimadzu, Kyoto, Japan), SIL-20AC autosampler (Shimadzu, Kyoto, Japan), a 6500 Q-Trap mass spectrometer (AB Sciex, Foster City, CA, USA), and an electrospray ionization (ESI) source (AB Sciex, Foster City, CA, USA).

The LC separations of analytes were performed on a reversed-phase ACE C_{18} column (5 cm × 2.1 mm ID, 3 μm particle size), which was coupled with a corresponding guard column (1 cm × 2.1 mm ID, 3 μm particle size; Advanced Chromatography Technologies, Aberdeen, UK). The mobile phase was prepared from two solvents: solvent A was 5 mM ammonium formate (pH = 3.5), and solvent B was acetonitrile. The gradient conditions were as follows: 2% solvent B for the first 0.1 min; 3.5 min, 90% solvent B; 6.0 min, 90% solvent B; 6.1 min, 2% solvent B; 10 min, 2% solvent B. The flow rate was set at 300 μL/min. The sample injection volume was 10 μL.

For mass spectrometric analysis, the source-dependent parameters were optimized by performing flow injection analysis (FIA) in positive mode. The ion-spray voltage was set at 5000 V, and source temperature was maintained at 500 °C. Curtain gas, gas 1, and gas 2 were 25, 40, and 40 psi, respectively. To select deprotonated molecule, we infused 1 μg/mL of each reference drug standard in full-scan mode. Compound-dependent parameters (declustering potential (DP), collision energy (CE), and collision cell exit–potential (CXP)) were optimized by manually tuning for each analyte. Table 1lists the DP, CE, and CXP values of each analyte. Multiple reaction monitoring (MRM) scan was performed to quantify 21 analytes. In this process, we identified the most intense product ion (PI) of each analyte. For all analytes, the entrance potential (EP) was 10 eV. For each ion transition, dwell time was 30 ms and pause time was 5 ms. Data acquisition and analysis were performed with Analyst 1.6.2 software (AB Sciex, Foster City, CA, USA).

Stability of 21 cephaloporins in zebrafish homogenate

We evaluated the stability of cephalosporin samples (25, 50, and 100 ng/mL concentrations), which were prepared in zebrafish homogenate according to the experiment conditions. For freeze-thaw stability, the samples were investigated after three cycles (−70 °C to 25 °C). For short-term stability, samples were kept at room temperature for 6 h. Long-term stability was measured by assaying the samples after storage at −70 °C for seven days. The average concentration of each analyte was determined from triplicate samples.