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Metabolism of SFZ-47 in chicken embryo by liquid chromatography-electrospray ion trap mass spectrometry[1]

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KEY WORDS SFZ-47; chicken embryo; metabolism; liquid chromatography; electrospray ionization mass spectrometry

ABSTRACT

AIM: To develop an alternative method for investigation of drug metabolism by fertilized chicken eggs using 3*H*-1,2-dihydro-2-(4-methyl-phenylamino) methyl-1-pyrrolizinone (SFZ-47) as a probe drug. **METHODS:** SFZ-47 (15 mg) was injected into the albumen of eggs from standardized breed chickens previously incubated for 10 d. After 72 h of further incubation, the allantoic liquid was subjected to solid phase extraction on XAD-2 columns and analyzed by liquid chromatography-electrospray ion trap mass spectrometry method. **RESULTS:** Three major metabolites were identified, namely 4-(3*H*-1,2-dihydro-1-pyrrolizinone-2-methyl-amino) benzyl alcohol (SFZ-47-OH), 4-(3*H*-1,2-dihydro-1-pyrrolizinone-2-methyl-amino)-benzoic acid (SFZ-47-COOH), and its glucuronide conjugates. The metabolic profile was little different from that previously found in rabbits and dogs. **CONCLUSION:** The result demonstrates the usefulness of the fertilized chicken egg as a convenient source of both phase I and phase II metabolites for further metabolism studies of SFZ-47.

INTRODUCTION

As part of the drug development process, studies of drug-metabolism are usually performed *in vivo* using animal models such as rats, rabbits, and dogs. In recent years, however, the use of *in vitro* model systems has greatly increased and now includes precision-cut liver tissue slices, primary cultures of hepatocytes, subcellular fractions, and heterologously expressed drug-metabolizing enzymes[1]. In the past, the fertilized chicken egg has been used to research the effects of drugs and hormones on fetal development, but its potential as a means of generating drug metabolites has not been exploited. Previous studies had demonstrated the presence of both phase I and II drug-metabolizing enzymes in chicken embryos[2-5], but there was little information related to the actual metabolites produced from specific drugs and the degree of species specificity involved[6].

3*H*-1,2-dihydro-2-(4-methyl-phenylamino) methyl-1-pyrrolizinone (SFZ-47) is a novel prodrug of an anti-inflammatory and analgesic agent in preclinical development. Previous studies have shown that it undergoes oxidative metabolism in the rabbits and dogs to 4-(3*H*-1,2-dihydro-1-pyrrolizinone-2-methyl-amino) benzyl alcohol (SFZ-47-OH) and 4-(3*H*-1,2-dihydro-

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1-pyrrolizinone-2-methylamino) benzoic acid (SFZ-47-COOH) followed by conjugation with glucuronic acid[7-9]. In this study, we used it as a probe drug to explore drug metabolism in fertilized chicken eggs as part of an assessment of the embryonated egg as a source of both phase I and phase II metabolites.

MATERIALS AND METHODS

Chemicals SFZ-47, SFZ-47-OH, and SFZ-47-COOH were kindly supplied by Shenyang Pharmaceutical University (Shenyang, China). The glucuronide of SFZ-47-COOH was isolated and purified in our laboratory[10]. Fertilized chicken eggs were obtained from a local hatchery. Methanol and acetonitrile were of HPLC grade. All other chemicals were of analysis grade.

Incubation and sample preparation Prior to injection, eggs were selected for fertility and normal development by candling. SFZ-47 (15 mg dissolved in 0.2 mL PEG-400) was injected into the albumen of eggs containing 10-d embryos through a small hole, which was then sealed with tape. Control eggs received solvent only. The eggs were incubated at 37 ºC and 60 % relative humidity for 72 h, and then subjected to -20 ºC for 30 min to stop hatching. The allantoic fluid was extracted with a syringe and centrifuged at 3000×*g* for 10 min. Sample preparation of the supernatant (10 mL) involved application to a preconditioned XAD-2 column (18 cm×2.2 cm) and washing with 20 mL water followed by 40 mL methanol at a flow rate of 2.0 mL/min. The methanol was collected and evaporated to dryness *in vacuum* at 40 ºC. The residue was dissolved in 1 mL methanol, filtered through a membrane (0.45 μm) and stored at -20 ºC until analysis.

LC-MS^n The HPLC system (Shimadzu Corp, Kyoto, Japan) consisted of a Shimadzu 10AD pump, a 7125 Rheodyne injector and a Kromasil ODS column (200 mm×4.6 mm, 5 μm, Hi-Tech Scientific Instrument Corp, Tianjing, China). The mobile phase was methanol: ammonium acetate 10 mmol/L (2:1, v/v, pH 4.5) at a flow rate of 0.4 mL/min. Ion trap-based LC-MS was performed using a Finnigan LCQ system (Finnigan Mat, San Jose, CA, USA) equipped with an atmospheric pressure ionization interface. The instrument was operated in the negative electrospray ionization mode directly coupled to the HPLC system via a Finnigan atmospheric pressure ionization source. The spray was generated using a sheath gas (N_2, 0.75 L/min) and an auxiliary gas (N_2, 0.15 L/min). MS^n spectra of precursor ions were obtained through incidental collision with neutral gas (He) molecules in the ion trap. The ionization was performed using the following parameters: spray voltage, 4.25 kV; capillary temperature, 150 ºC; capillary voltage, -30 V. Spectra were collected in the mass range from *m/z* 100 to 500. Data were collected and analyzed using Navigator software version 1.2 (Finnigan).

Stability of SFZ-47 and its major metabolites To validate the sample preparation procedure, SFZ-47, SFZ-47-OH, SFZ-47-COOH, and its glucuronide conjugate were added to freshly collect allantoic fluid at concentrations of 10 mg/L and allowed to stand at room temperature (<25 ºC) for 10 h. Each sample was then extracted and analyzed by LC-MS^n as described above.

RESULTS

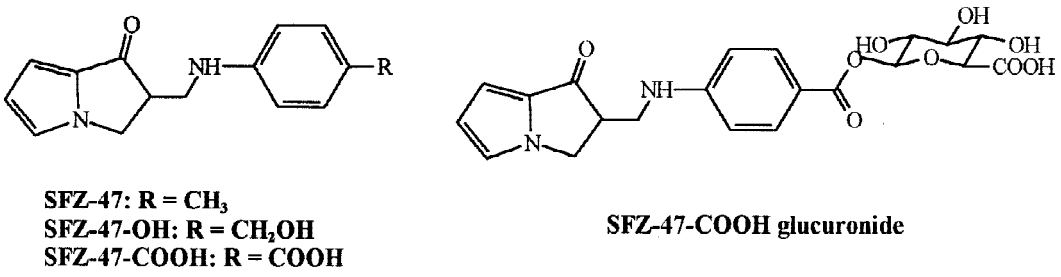
According to the structure of SFZ-47 and its assumed metabolites, their mass spectra were detected in positive and negative model, respectively. Compared with the blank sample, (+) ESI full scan mass spectra of samples from the allantoic fluid in embryonated eggs injected SFZ-47 provided protonation molecular ions $[M+H]^+$ at *m/z* 241, and (-) ESI mass spectra provided pesudomolecular ions $[M-H]^-$ at *m/z* 255, 269, and 445. The selected ions monitoring (SIM) chromatograms were illustrated in Fig 1. The chromatogram and mass spectra of *m/z* 241 corresponded to reference standard of SFZ-47 (Fig 2). Metabolites were identified by MS^2 and MS^3 spectra.

M1: MS^2 spectra of M1 (*m/z* 269) provided characteristic fragment ions at *m/z* 120 and 148. It was thus identified as SFZ-47-COOH confirmed by comparison of its mass spectra with that of the reference standard (Fig 3).

M2: MS^2 spectra of M2 (*m/z* 445) gave daughter ions at *m/z* 269 and 175. The MS^3 spectra of *m/z* 269 provided fragment ions at *m/z* 120 and 148, being consistent with that of SFZ-47-COOH. The MS^3 spectra of *m/z* 175 gave daughter ions at *m/z* 113 consistent with lose CO_2 and H_2O from glucuronic acid[11,12]. The chromatogram and MS^n spectra of the reference standard confirmed M2 as the glucuronide conjugate of SFZ-47-COOH (Fig 4).

M3: MS^2 spectra of M3 (*m/z* 255) provided characteristic fragment ions at *m/z* 120 and 134. It was thus identified as SFZ-47-OH confirmed by comparison of its mass spectra with that of the reference standard (Fig 5).

LC-MS^n was used to examine the stability of SFZ-



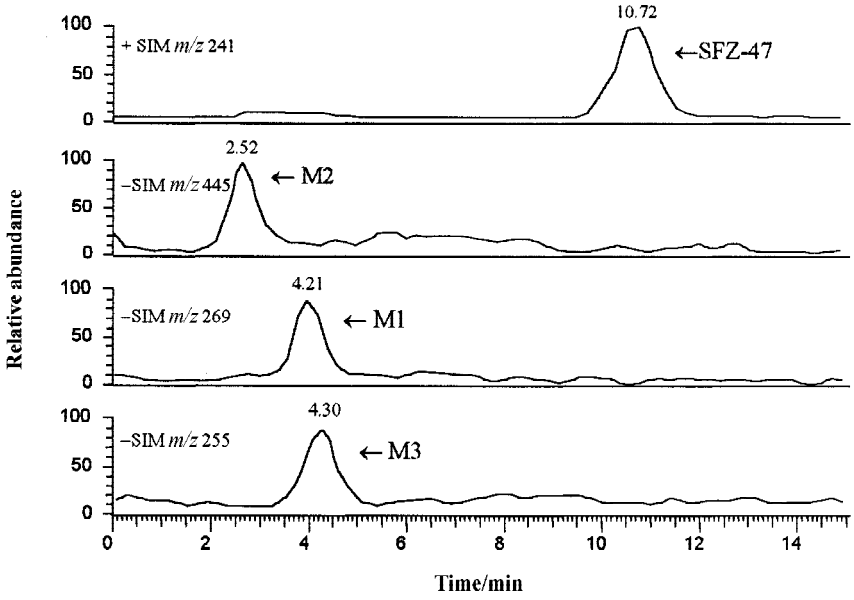


Fig 1. SIM chromatograms of SFZ-47 and its metabolites in the allantoic liquid of chicken embryos.

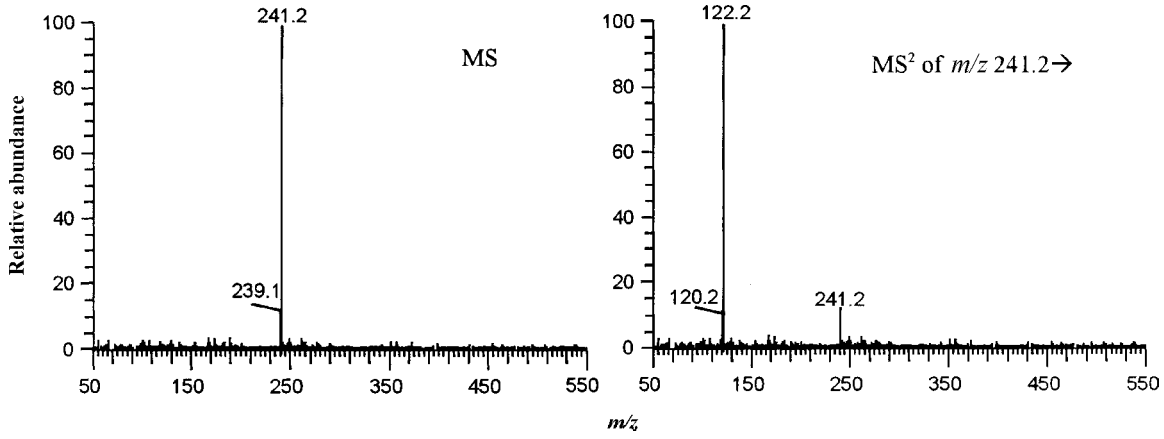


Fig 2. MS^n of SFZ-47 in the allantoic liquid.

47 and its major metabolites in allantois liquid of fertilized chicken eggs. After standing at room temperature for 10 h, no degradation product of each substance above in the spiked biological fluids was observed. This indicated that under the current experimental conditions, SFZ-47 and its major metabolites were stable.

DISCUSSION

This preliminary study was to investigate the use of the fertilized chicken egg as a model system for the generation of drug metabolites. A drug known to un-

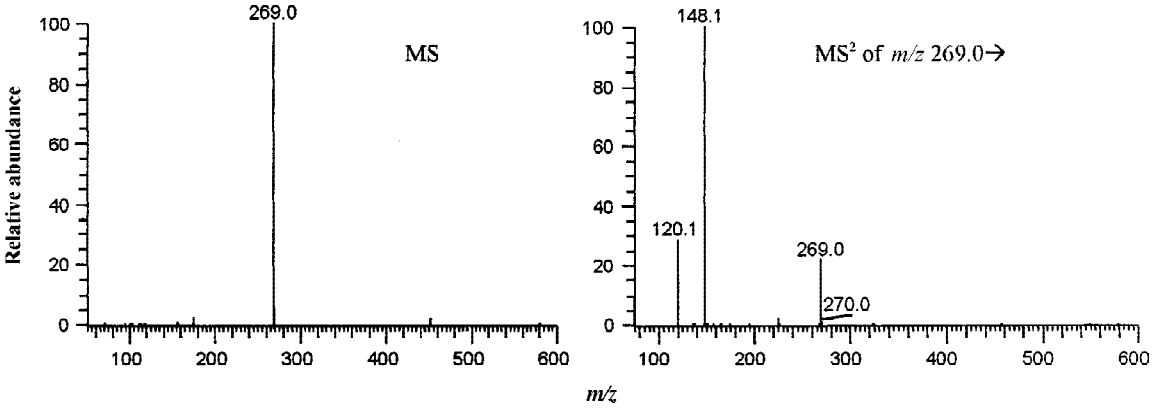


Fig 3. MS^n of SFZ-47-COOH (M1) in the allantoic liquid.

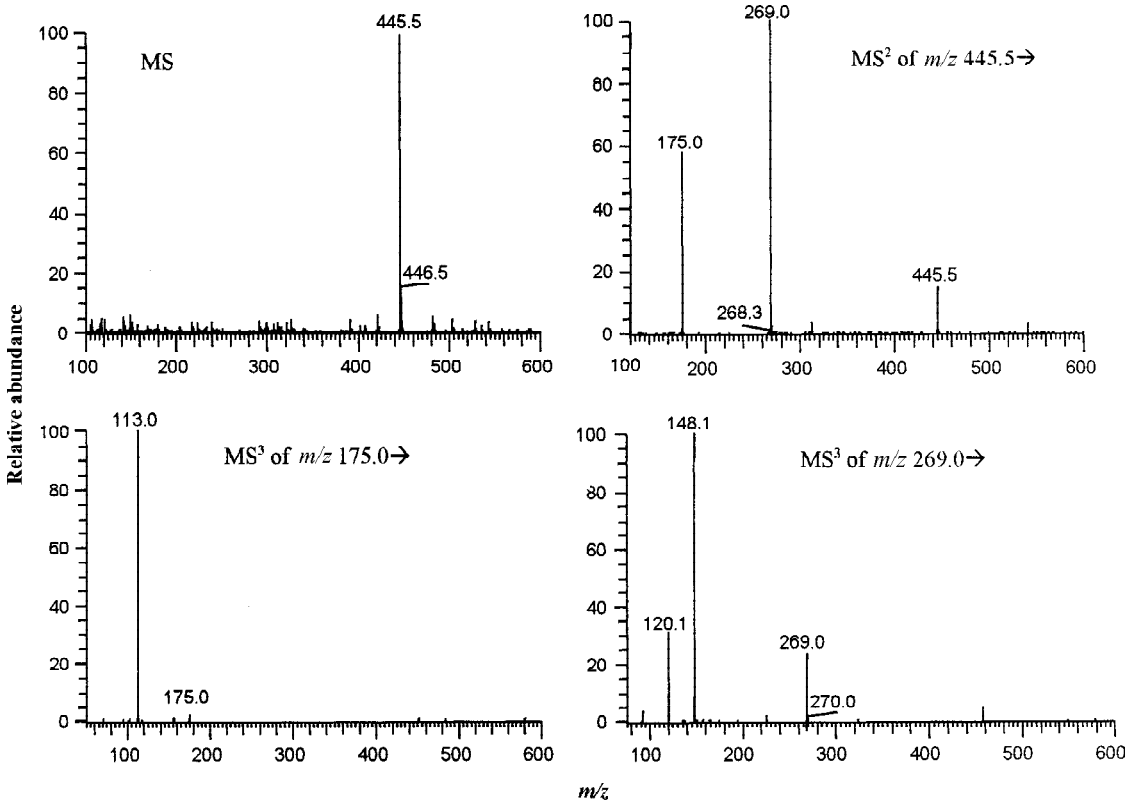


Fig 4. MS^n of SFZ-47-COOH glucuronide (M2) in the allantoic liquid.

dergo phase I and II metabolism in the rabbits and dogs was used as a probe. SFZ-47 and its metabolites were identified in the allantois fluid by LC-MS^n and specific ion monitoring. The major metabolic pathways of SFZ-47 involved oxidation followed by glucuronidation (Fig 6). These paths are also found in the rabbits and dogs, but glucuronide of SFZ-47-OH was not detected in the embryonated eggs. Although Neugebauer M indicated that the preference for sulfation appeared to be a feature of chicken embryo metabolism[6], we did not find such species difference in this study.

Compared with other *in vitro* models of drug metabolism, the fertilized chicken egg is characterized by relatively low cost, ease of use, freedom from maternal influences, and amenability to environmental control. As an intermediate step between *in vitro* and *in vivo* models, the embryonated egg will be applied to drug metabolism studies.

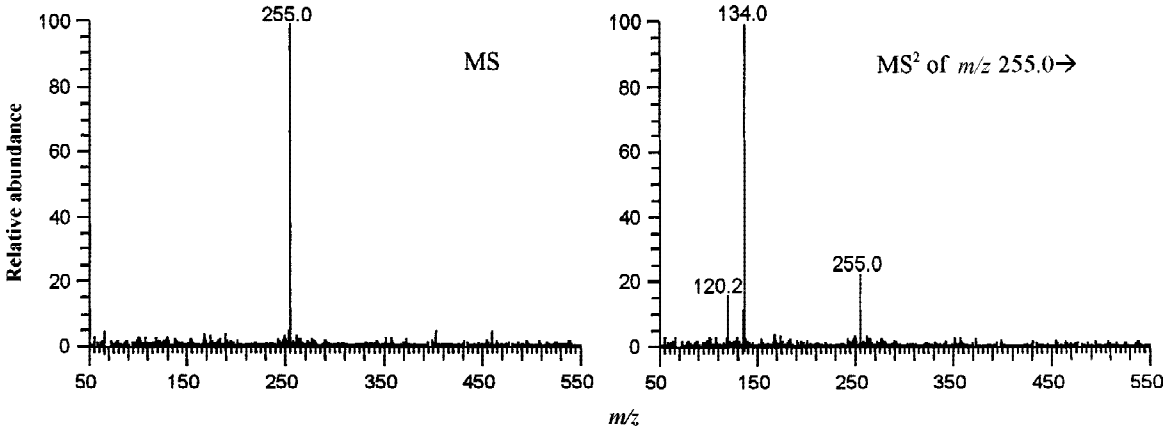


Fig 5. MS^n of SFZ-47-OH (M3) in the allantoic liquid.

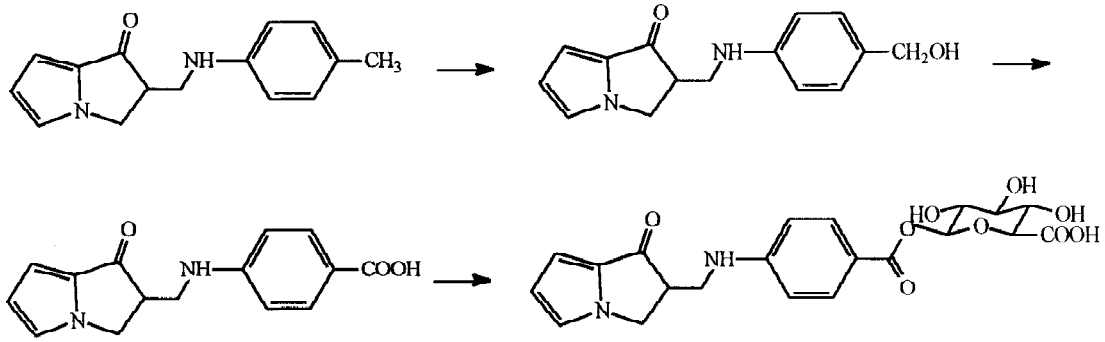


Fig 6. The proposed major metabolic pathway of SFZ-47 in the chicken embryo.

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