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Short communication

Isolation and identification of the glucuronide of 4-(3*H*-1,2-dihydro-1-pyrrolizinone-2-methylamino)benzoic acid from rabbit urine

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Abstract

The metabolic profile of 3*H*-1,2-dihydro-2-(4-methylphenylamino)methyl-1-pyrrolizinone (SFZ-47), a putative non-steroidal anti-inflammatory pro-drug, has been studied in rabbit urine. Semi-preparative reversed-phase HPLC of 24 h urine from two rabbits given single oral doses of SFZ-47 (200 mg) allowed the separation of SFZ-47 together with the oxidative metabolite 4-(3*H*-1,2-dihydro-1-pyrrolizinone-2-methylamino)benzoic acid (SFZ-47-COOH) and its glucuronide conjugate. The glucuronide was characterized by ESI-MS^n and ^1H NMR and shown to be the 1-*O*-acyl β-D-glucuronide conjugate of SFZ-47-COOH. The method gave excellent resolution of the glucuronide from endogenous constituents in urine and may be suitable for the preparation of glucuronide metabolites of other drugs.

Keywords: 4-(3*H*-1,2-Dihydro-1-pyrrolizinone-2-methylamino)benzoic acid glucuronide

1. Introduction

For many drugs and their metabolites possessing –OH, –SH, –NHR or –COOH groups, conjugation with endogenous glucuronic acid to yield water soluble products is the major pathway in their biotransformation and elimination from the body. Glucuronides are generally considered to be inactive and rapidly excreted but in recent times many highly active or toxic glucuronides of drugs have been found in vivo (e.g. morphine-6-glucuronide is 45 times more potent than the parent drug) [1,2]. Thus, it is important to re-evaluate the biological significance of glucuronidation and the pharmacological activity of glucuronides. 3*H*-1,2-dihydro-2-(4-methylphenylamino)methyl-1-pyrrolizinone (SFZ-47) is a novel pro-drug of an anti-inflammatory and analgesic agent in pre-clinical development. Previous studies have shown that it undergoes oxidative metabolism in the rabbit to 4-(3*H*-1,2-dihydro-1-pyrrolizinone-2-methylamino)benzoic acid (SFZ-47-COOH) followed by glucuronidation (Fig. 1) [3]. Acyl glucuronides are difficult to synthesize because they undergo hydrolysis and intramolecular acyl migration at physiological pH [4–6]. In this study, the glucuronide of SFZ-47-COOH has been isolated from rabbit urine by semi-preparative reversed-phase HPLC and fully characterized by mass spectroscopy

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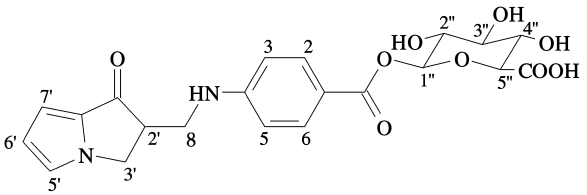

Fig. 1. The structure of glucuronide conjugate of SFZ-47-COOH.

and NMR spectroscopy. The glucuronide conjugate is now available as an authentic standard for use in pharmacokinetic and pharmacodynamic studies.

2. Experimental

2.1. Chemicals and reagents

SFZ-47 and SFZ-47-COOH were kindly supplied by Shenyang Pharmaceutical University (Shenyang, China). Acetonitrile was HPLC grade. All other chemicals were analysis grade and used without further purification.

2.2. Samples preparation

Two male rabbits (obtained from department of experimental animals of Jilin University) weighting 2.0 kg were housed in metabolism cages for the collection of urine. They were administered 200 mg intragastric gavage doses of SFZ-47 dissolved in PEG-400. Urine was collected for 24 h, adjusted to pH 3.5 with acetic acid and lyophilized (EYEV4, Tokyo Rikakikai, Japan). The residues were reconstituted in 20 ml methanol and centrifuged at 2500 *g* for 10 min. The supernatant was filtered (0.45 μm) and then stored at −20 °C until analysed by HPLC.

2.3. Chromatography

The HPLC system (Waters, USA) consisted of a 515 pump, a 7725i Rheodyne injector and a 2487 UV detector. Samples were analyzed on a PrepNova-Pak C_{18} radial compression column (particle size, 6 μm; 200×25 mm I.D., Waters, USA) using a solvent gradient. Mobile phase A was acetonitrile, and mobile phase B was water adjusted to pH 3.5 with acetic acid. The elution gradient was from A/B 20/80 to 40/60 over 30 min followed by 40/60 to 60/40 over 15 min. Other parameters were as follows: flow-rate, 8.0 ml/min; detection wavelength, 290 nm; injection volume, 500 μl; column temperature, ambient temperature. The eluent collected from individual peaks was evaporated by rotary evaporation and freeze-drying. The compound was then identified with ESI-ion trap mass spectrometry and ^{1}H NMR spectroscopy.

2.4. Mass spectrometry

Mass spectra were obtained by using a quadrupole ion trap mass analyzer fitted with an ESI source (LCQ, Finnigan-MAT, San Jose, USA) in the negative ion mode. Each sample was dissolved in methanol at a concentration of 50 ng/ml and delivered using direct infusion with a syringe pump at a flow-rate of 10 μl/min. The interface was operated with the heated capillary at a temperature of 150 °C, and with a spray voltage of 4.5 kV. The sheath gas and auxiliary gas (both were nitrogen) flows were set at 28 and 3 units (instrument settings in arbitrary units), respectively. In MS^n (n=2–3) experiments, collision-induced dissociation (CID) was carried out in the trap on a selected ion by transferring energy via a radio frequency potential applied to the end-cap electrodes. The collision energy was chosen that allowed a sufficient fragmentation of the $[M-H]^-$ or the most abundant fragment ion.

2.5. NMR spectroscopy

^{1}H NMR spectra were measured on a Unity 400 superconductive spectrometer operating at 399.94 MHz (Varian, USA). Free induction decays (FIDs) were collected into 16 K computer data points with a spectral width of 5104.0 Hz, 90° pulses were used with an acquisition time of 4.0 s, and the spectra were acquired by accumulation of 128 scans.

3. Results

The reversed-phase HPLC profile of 24 h rabbit urine after administration of SFZ-47 is illustrated in Fig. 2. Three peaks were clearly resolved from each other and from endogenous polar compounds in urine. The peaks in order of elution were assigned to the glucuronide conjugate of SFZ-47-COOH (peak 1), SFZ-47-COOH (peak 2) and SFZ-47 (peak 3).

The full scan MS of peak 1 gave an abundant pseudomolecular ion $[M-H]^-$ at m/z 445. The MS^2 of m/z 445 showed daughter ions at m/z 269 and 175. The MS^3 of m/z 269 gave fragment ions at m/z 120 and 148 as found with SFZ-47-COOH; the MS^3 of m/z 175 gave a fragment ion at m/z 113 diagnostic of glucuronic acid (Fig. 3) [7]. Based on these data, peak 1 is identified as the glucuronide of SFZ-47-COOH.

Further characterization of the glucuronide as the1-*O*-acyl β-D-glucuronide of SFZ-47-COOH was carried out by ^{1}H NMR spectroscopy [8]. To prevent interference from –OH groups on the sugar ring, the sample was dissolved in D_2O. Proton chemical shifts were observed at: 3.44 (1H, m, 2′e-H), 3.4~3.6 (3H, m, 2″, 3″, 4″-H), 3.53 (2H, m, 8-H), 3.83 (1H, d, J=9.2 Hz, 5″-H), 4.08 (1H, dd, J=3.2 Hz, 12.4 Hz, 3′e-H), 4.38 (1H, dd, J=7.6 Hz, 12.4 Hz, 3′a-H), 5.62 (1H, d, J=7.2 Hz, 1″-H), 6.50 (1H, dd, J=2.0 Hz, 4.0 Hz, 6′-H), 6.61 (2H, d, J=8.8 Hz, 3-H, 5-H), 6.68 (1H, d, J=4.0 Hz, 7′-H), 7.19 (1H, br.s, 5′-H), 7.76 (2H, d, J=8.8 Hz, 2-H, 6-H).

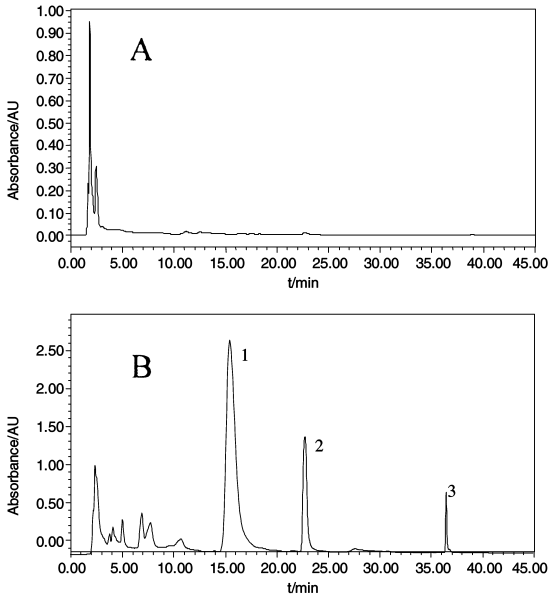

Fig. 2. Semi-preparative HPLC of rabbit urine. (A) blank urine; (B) 0~24 h urine of a rabbit receiving 200 mg of SFZ-47; (1) glucuronide conjugate of SFZ-47-COOH; (2) SFZ-47-COOH; (3) SFZ-47.

4. Discussion

Because labile acyl glucuronides are difficult to synthesize, it is usual to obtain them by a biosynthetic method. Such methods include isolation from human or animal urine after dosing with the parent compound, synthesis using enzyme mixtures derived from mammalian tissue or synthesis using immobilized enzyme mixtures [4]. As the first method is simple and economical, we adopted it in this study to prepare the glucuronide of SFZ-47-COOH.

In general, glucuronides are extracted from biofluids using ethyl acetate [9] or XAD-2 resin [10]. However, in the case of SFZ-47-COOH glucuronide in urine, the amount extracted with ethyl acetate proved to be inadequate and the use of XAD-2 resin required the removal of large volumes of aqueous eluent. Reversed-phase HPLC of concentrated urine proved to be the most efficient procedure.

Because glucuronides are highly water soluble, efficient separation from endogenous polar material by reversed-phase HPLC requires gradient elution. Good resolution of the glucuronide and SFZ-47-COOH was obtained by an initial slow increase in the fraction of organic solvent in the mobile phase. Elution of the more lipophilic SFZ-47 was then achieved using a more rapid increase in the fraction of organic solvent. No positional isomers resulting from acyl migration were observed by ^{1}H NMR analysis, because the urine sample and mobile phase of HPLC were acidified to pH 3.5.

In conclusion, a rapid and simple approach for preparing an acyl glucuronide of SFZ-47-COOH has been developed. We are currently applying this approach to the preparation of other *N*- and *C*-glucuronides as part of a systematic attempt to

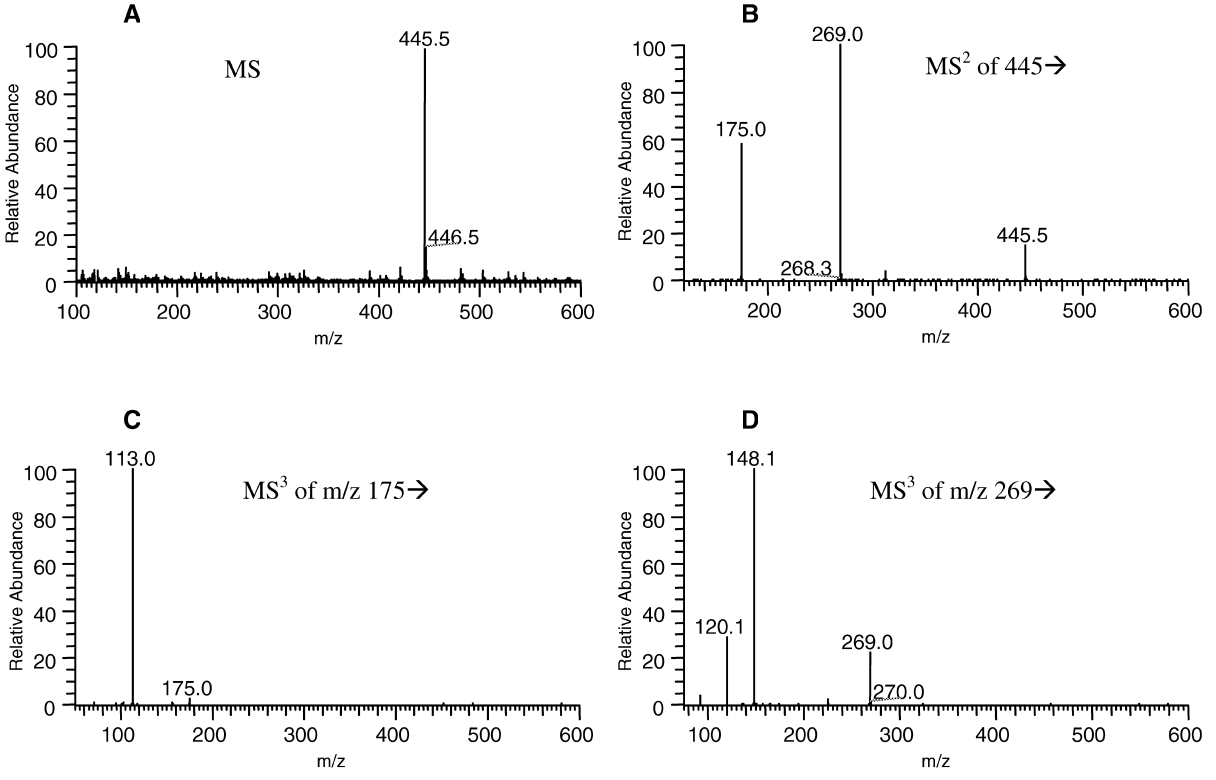

Fig. 3. MS^n spectra for the glucuronide conjugate of SFZ-47-COOH.

explore the general principles involved in the separation and characterization of glucuronide conjugates.

Acknowledgements

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