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Research Article

Metabolic Study of Stable Isotope Labeled Indolinone Derivative in Hepatocyte Cell by UPLC/Q TOF MS

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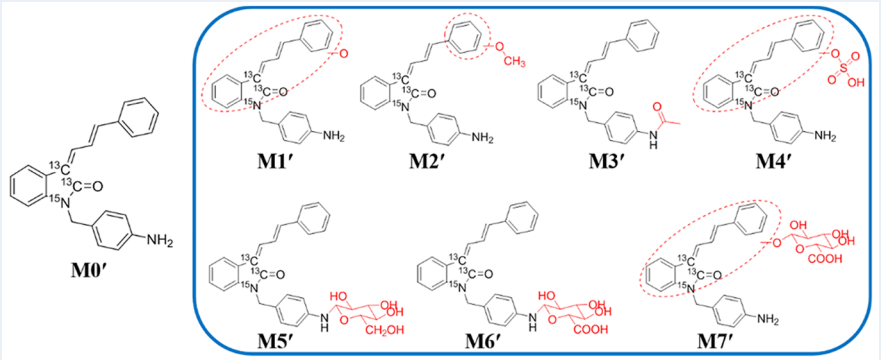

ABSTRACT: The aggregation process of α-synuclein (α-syn) is substantial in the pathogenesis of Parkinson's disease. Indolinone derivatives are inhibitors of α-syn aggregates and can be used as PET-based radiotracers for imaging α-syn fibrils. However, no investigations on the metabolism of indolinone derivatives have been reported until now. In the present research, a ^{13}C and ^{15}N isotope labeling strategy was developed to synthesize compound [$^{13}C_2$,^{15}N]-(*Z*)-1-(4-aminobenzyl)-3-((*E*)-(3-phenyl)allylidene)-indolin-2-one (**M0′**), which was then used in a study of metabolism in hepatocytes. The metabolites were characterized using accurate mass and characteristic ion measurements. In the metabolic system, compound **M0′** was the main component (accounting for 97.5% of compound-related components) after incubation in hepatocytes for 3 h, which indicated that compound **M0′** possessed great metabolic stability. Seven metabolites have been successfully verified by UPLC/Q TOF MS in metabolic studies, including hydroxyl **M0′** (**M1′**), hydroxyl and methylated **M0′** (**M2′**), *N*-acetylated **M0′** (**M3′**), sulfate of hydroxyl **M0′** (**M4′**), the glucose conjugate of **M0′** (**M5′**), glucuronide conjugate of **M0′** (**M6′**), and glucuronide conjugate of hydroxyl **M0′** (**M7′**). The study on metabolism provides the important information to develop effective α-syn aggregate inhibitors and new PET-tracer-related indolinone derivatives.

KEYWORDS: *indolinone derivatives, α-synuclein, [$^{13}C_2$, ^{15}N]-isotope labeling, metabolite, UPLC/Q TOF MS*

INTRODUCTION

The metabolites generated *in vivo* have similar structures to the parent drug and may possess potential pharmacological activity or toxicity, so it is essential to identify metabolites in the early stage of drug development.[1−4] The liver is the most important organ for metabolism in humans, and the hepatocytes contain more metabolic enzymes compared with liver microsomes, liver cytosolic fractions, and pooled human liver S9 fractions and also can allow small-molecular-weight drugs (MW ∼ 500 Da) to enter in a variety of ways, such as passive diffusion, active transport of transporters, and so on.[5−7] Metabolic studies are therefore usually carried out *in vitro* with human hepatocytes before clinical studies.

It is crucial to identify metabolites for drug development, but it is also difficult to detect and predict the presence and nature of unknown metabolites due to endogenous interference from proteins and lipids.[8−10] Stable-isotope-labeled drugs could retain the requisite physical and chemical properties but have different molecule weights, which is conducive to detection by mass spectrometry.[11−13] Stable isotope labeling is an effective method of identifying drug metabolites.[14−16] Based on the elements found in many drugs, stable isotopes ^{2}H, ^{13}C, ^{15}N, and ^{18}O are usually candidates.[17,18] However, significant kinetic isotope effects with ^{2}H may affect, or even change, the metabolic pathway of drugs, and the ^{18}O of any carbonyl group might be exchanged with the ^{16}O in H_2O in the form of harmonic alcohol.[9,19] Thus, ^{13}C and ^{15}N are more suitable for

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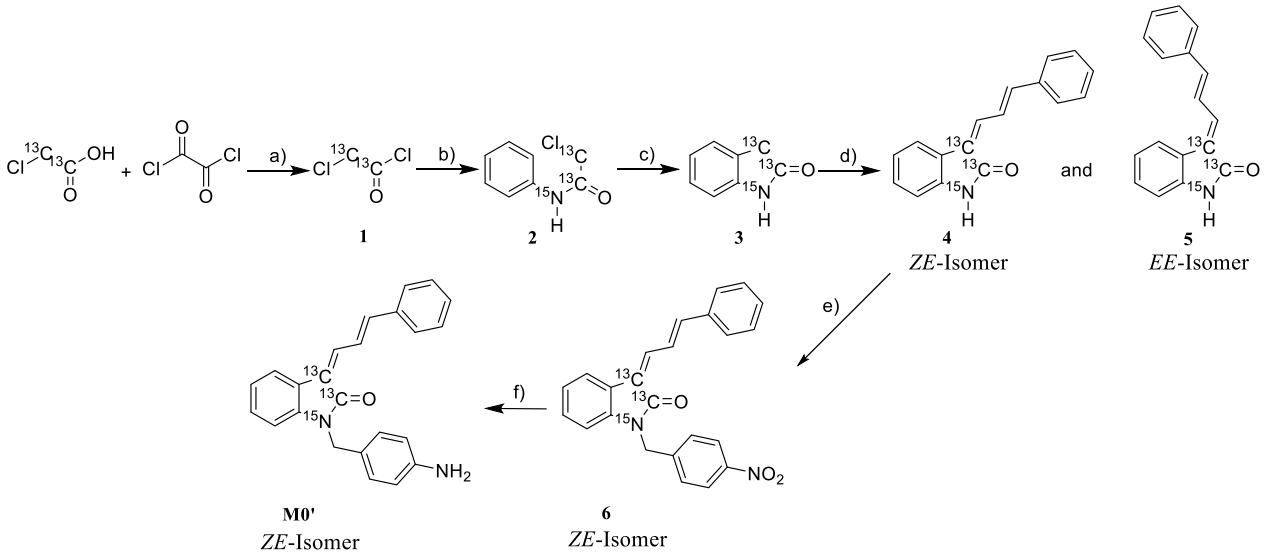
Scheme 1. Synthesis of M0′[a]



[a]Key: (a) DCM, DMF, 0 °C to rt, 4 h; (b) [^{15}N]-aniline, DCM, triethylamine, 0 °C to rt, 1 h; (c) anhydrous alchlor, 230 °C, 15 min; (d) cinnamaldehyde, piperidine, ethanol, reflux, 1 h; (e) 4-nitrobenzyl bromide, 60% NaH, DMF, rt, 0.5 h; (f) Fe/HCl, methanol, reflux, 2 h.

use in stable isotope labeling than ^{2}H and ^{18}O for the identification of metabolites.

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder, which threatens approximately 2% of the population over the age of 60.[20−22] The aggregation of α-syn is key to PD pathogenesis.[23,24] Indolinone derivatives showed activity and selectivity toward α-syn fibrils as demonstrated by Mach et al. Furthermore, the indolinone analogues were also developed as PET-based radiotracers for imaging α-syn fibrils.[25] Although indolinone derivatives showed potential in the treatment of PD, the metabolite study of indolinone derivatives has never been reported before. (*Z*)-1-(4-Aminobenzyl)-3-((*E*)-(3-phenyl)-allylidene)indolin-2-one (**M0**) is our candidate compound developed for treatment of PD, which can inhibit α-syn fibrils to a significant extent in terms of measured activity (data not published). The present study is to synthesize and characterize the stable labeled indolinone derivative [$^{13}C_2$,^{15}N]-(*Z*)-1-(4-aminobenzyl)-3-((*E*)-(3-phenyl)allylidene)indolin-2-one (**M0′**) and then profile the metabolites in human hepatocytes using the stable labeled compound by UPLC/Q TOF MS.

EXPERIMENTAL SECTION

Chemicals and Reagents. [$^{13}C_2$]-Chloroacetic acid with isotopic enrichment for 99% and [^{15}N]-aniline with isotopic enrichment for 100% were purchased from Cambridge Isotope Laboratories (Andover, MA). Cryopreserved human hepatocytes for the metabolite incubation were obtained from BD Gentest (Woburn, CA). All solvents for the ultraperformance liquid chromatography (UPLC)/quadrupole time-of-flight mass spectrometry (Q TOF MS) analyses were of high-performance liquid chromatography grade (Merck, Darmstadt, Germany). Ultrapure water was generated using a Milli-Q Gradient system (Millipore Corp., Molsheim, France). Other reagents were analytical grade and used without further purification.

Synthesis of Compound M0′. As shown in Scheme 1, compound **M0′** was prepared through a six-step reaction with [$^{13}C_2$]-chloroacetic acid and [^{15}N]-aniline as starting materials. The preparations of compounds **1**−**3** were described in our previous report.[26] The detailed synthetic steps for compounds **4**−**6** and **M0′** are shown in S1. Compounds **4**−**6** and **M0′** were purified by column chromatography, and the structures of compounds **4**−**6** and **M0′** were further confirmed by ^{1}H NMR and HRMS (Figures S1−S4). The isotopic enrichment in compound **M0′** was approximate 99%.

***In Vitro* Samples Incubation and Pretreatment.** Hepatocytes metabolism tests *in vitro* were carried out by undergoing the method from ref 7. Briefly, the hepatic cell line was cultured in William's Medium E (pH 7.4) at a density of 1.0×10^6 cells/mL under a volume of 100 μL. Hepatocytes were treated with 10 μM labeled or unlabeled compound in a 37 °C incubator. After 3 h, cold acetonitrile (200 μL) was added to quench the reaction. The test samples were stored at −70 °C.

The incubated hepatocytes were vortexed and centrifuged at 14000 rpm for 5 min. The supernatant was completely collected and blown dry with nitrogen stream at 40 °C. The dried residues were redissolved in acetonitrile−water (200 μL, 50:50, v/v). The labeled and unlabeled samples were taken to analyze by UPLC/Q TOF MS.

UPLC/Q TOF MS Analysis. Chromatographic separation for the metabolite profiling and identification were conducted on an Acquity UPLC HSS T3 column (100 × 2.1 mm, i.d., 1.8 μm) at 45 °C using an Acquity UPLC system (Waters, Milford, MA). The mobile phase consisted of 0.02% formic acid in ammonium acetate (5 mM, elution phase A) and acetonitrile (elution phase B). The elution duration was set as 30 min, and elution gradient was as follows: 90% A−10% B from 0 to 20 min, 30% A−70% B from 20 to 25 min, 10% A-90% B from 25 to 27 min, 10% A-90% B from 27 to 30 min.

MS detection was achieved using a quadrupole TOF 5600+ MS/MS system (AB Sciex, Concord, Ontario, Canada) in the positive electrospray ionization (ESI) mode. The mass range was set at *m*/*z* 50−1000. The parameters were set as follows: ion spray voltage, 5500 V; declustering potential, 60 V; ion source heater, 550 °C; curtain gas, 40 psi; ion source gas 1, 55 psi; and ion source gas 2, 55 psi. For the TOF MS scans, the

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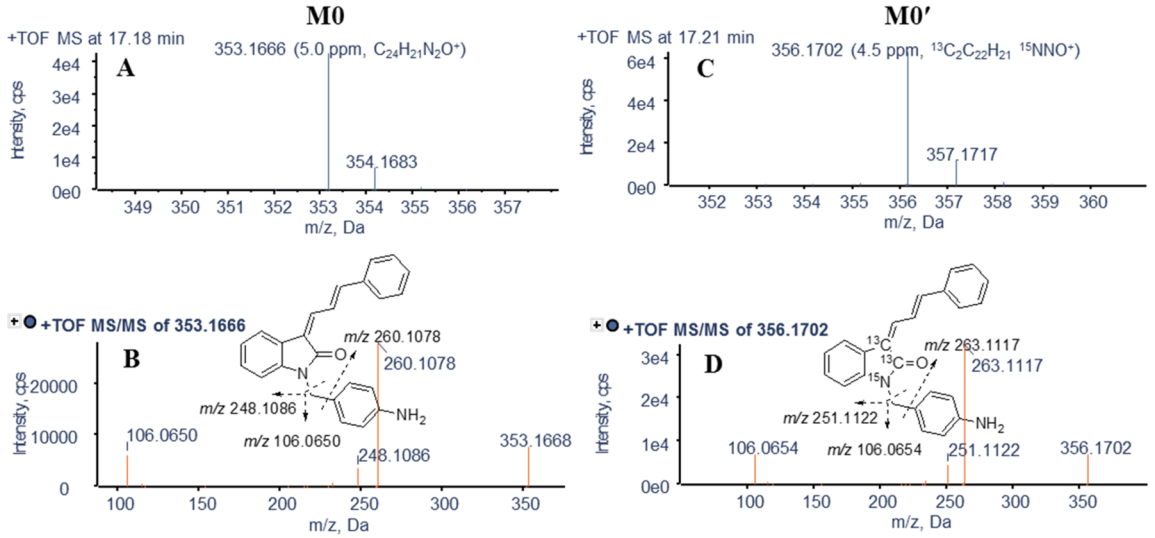

Figure 1. MS and MS/MS spectra of **M0** and **M0′**. (A) MS spectrum of **M0**, (B) MS/MS spectrum of **M0**, (C) MS spectrum of **M0′**, (D) MS/MS spectrum of **M0′**.

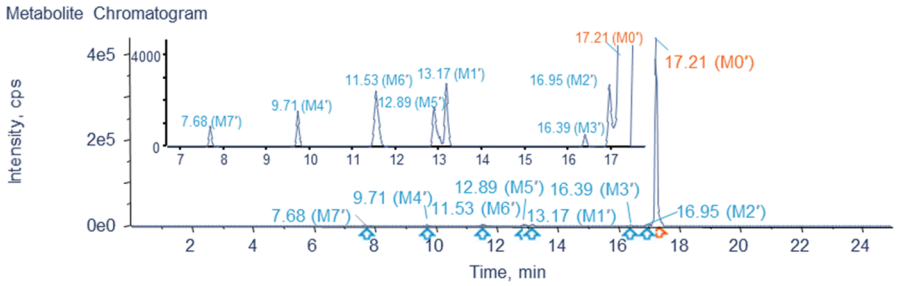

Figure 2. Metabolites profiling of **M0′** after incubation with human hepatocytes for 3 h at 37 °C by UPLC/Q TOF MS. The inset is the expanded chromatogram in the region of 7−18 min.

collision energy was 10 eV. For product ion scans, the collision energy was 15 eV, with a spread of 15 eV in the MS/MS experiment. Information-dependent acquisition (IDA) was used to trigger the acquisition of MS/MS spectra for ions matching the IDA criteria. A real-time multiple mass defect filter was used for the IDA criteria. The automatic calibration delivery system (CDS) was used to correct of MS and MS/MS by external standard method. The resolutions for MS or MS/MS analysis were maintained above 30000 or 15000−20000.

Data Analysis and Metabolites Identification. Metabolites were identified and characterized by Metabolite Pilot 2.0 software with multiple postprocessing functions. Comparing of the raw data, we could acquire the information on probable metabolites products, such as biotransformation, molecular formula, retention time, *m/z* values, and relative error (ER) by Metabolite Pilot 2.0 software (AB Sciex, USA). According to the full-scan MS spectra and MS/MS spectra of the probable metabolites and special focus on the mass shift resulted from stable labeling, the structure characterization of compound **M0′** was analyzed and the probable chemical structures of the metabolites were proposed.

RESULTS AND DISCUSSION

Strategy for Metabolite Discovery and Identification. The identification of metabolites in complex biological matrices is challenging due to the presence of an excess of proteins, lipids, and other endogenous materials which potentially interfere with the detection of drug-derived material. Therefore, the [$^{13}C_2$,^{15}N] isotope labeling strategy was developed to aid in the detection and identification of the metabolites of the indolinone derivative. Both labeled and unlabeled compounds were treated with hepatocytes, respectively. The molecular formulas of potential metabolites could be determined according to accurate high-resolution full scan mass data. Then the observed product ions were initially compared with those of parent compounds to assess structural similarities and changes within the metabolites. These tests were performed with the aid of the mass difference resulting from the stable-isotope labeling.

First, the standards of compounds **M0′** and **M0** were analyzed by UPLC/Q TOF MS in positive-ion mode. Figure 1 shows the MS and MS/MS spectra of compounds **M0** and **M0′**, in which **M0** and **M0′** gave a protonated molecular ion at *m/z* 353.1666 and 356.1702, indicating that their elemental compositions were $C_{24}H_{21}N_2O$ and $^{13}C_2C_{22}H_{21}{}^{15}NNO$, respectively. The unlabeled compound **M0** produced three major characteristic product ions at *m/z* 260.1078, 248.1086, and 106.0650; the labeled compound **M0′** produced corresponding product ions at *m/z* 263.1117, 251.1122, and 106.0654. The product ions at *m/z* 263.1117 and 251.1122 were formed by the loss of the aniline moiety and the *p*-toluidine group, respectively. The product ion at *m/z* 106.0654 was obtained from the *p*-toluidine group.

After incubation with human hepatocytes, the metabolites of compounds **M0′** and **M0** were detected by UPLC/Q TOF MS and seven metabolites were identified (Figures 2 and S5,

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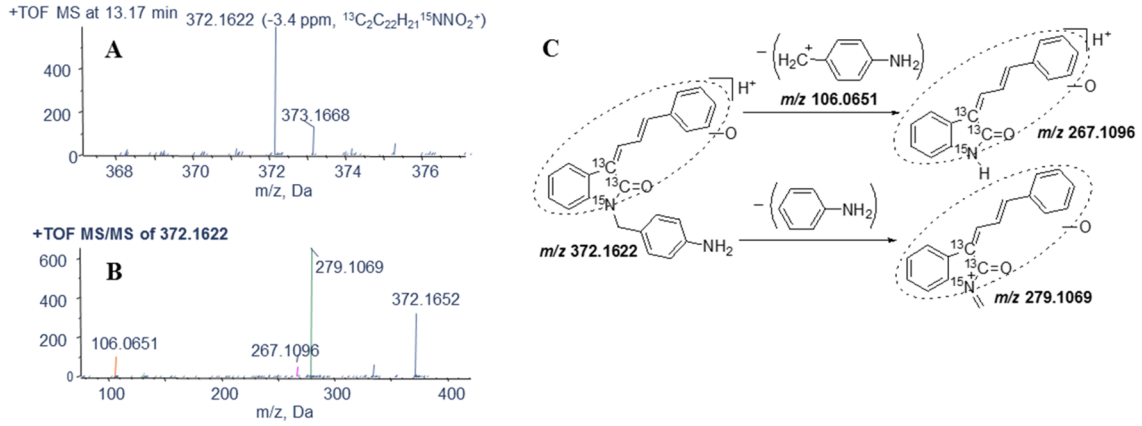

Figure 3. MS (A), MS/MS spectra (B), and fragmental pathways (C) of **M1′**.

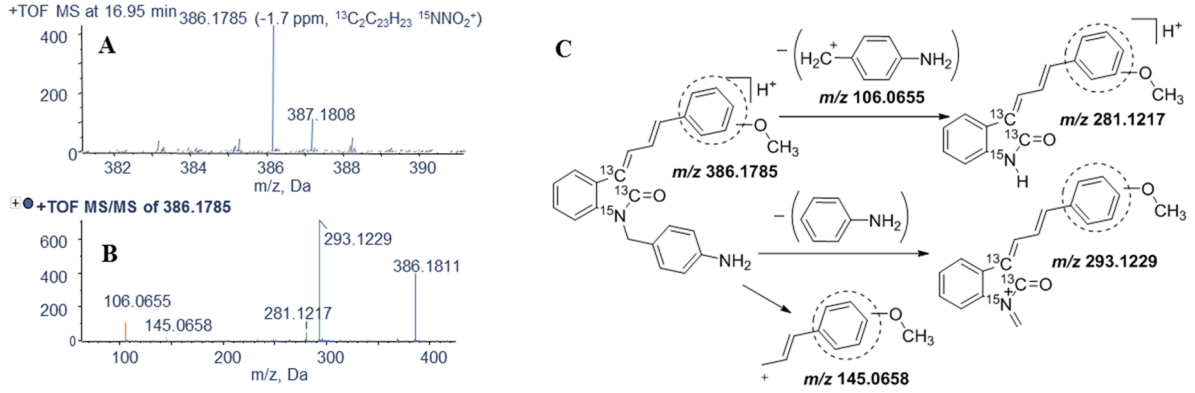

Figure 4. MS (A), MS/MS spectra (B), and fragmental pathways (C) of **M2′**.

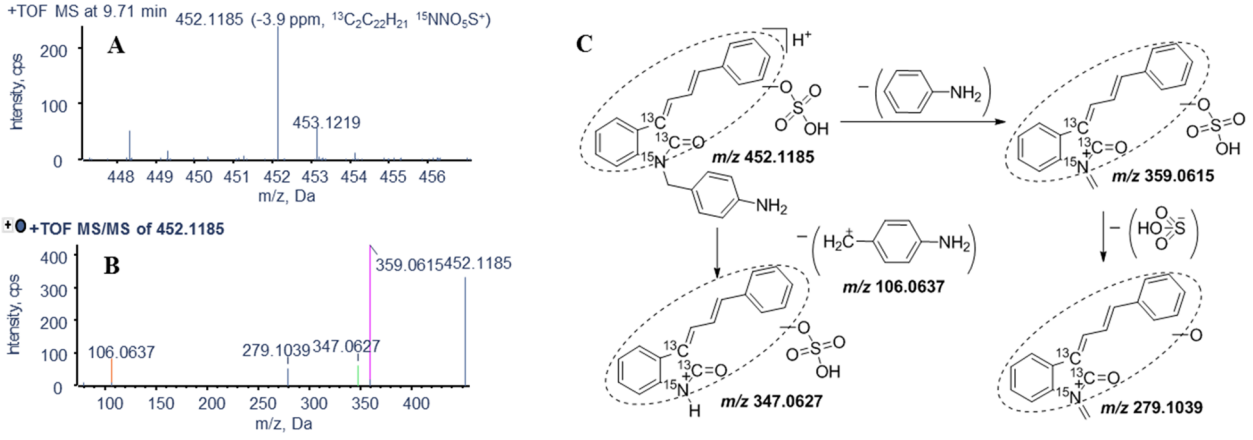

Figure 5. MS (A), MS/MS spectra (B), and fragmental pathways (C) of **M4′**.

respectively). **M1′** was eluted at 13.17 min, with a protonated molecular mass of *m/z* 372.1622 (Figure 3). The elemental composition of **M1′** was $^{13}C_2C_{22}H_{21}{}^{15}NNO_2$, indicating the addition of an oxygen to **M0′**. The fragment ion of **M1′** at *m/z* 106.0651 was the same as that of **M0′**. The fragment ions at *m/z* 279.1069 and 267.1096 were 16 Da bigger than those of **M0′**. According to the proposed fragmental pathways, **M1′** could be formed through the aromatic hydroxylation or the oxidation of the alkene to an epoxide. However, the sulfate of hydroxyl **M0′** (**M4′**) and the glucuronide of hydroxyl **M0′** (**M7′**) were detected as downstream metabolites of **M1′**; therefore **M1′** was proposed to be the phenol metabolite of **M0′**, but the precise oxidative location could not be determined. As expected, **M1** formed from **M0** was also detected with the protonated molecular mass at *m/z* 369.1580 (a mass-shift of 3 Da from *m/z* 372.1622) and the product ions at *m/z* 276.1032 and 264.1016 (a mass-shift of 3 Da from *m/z* 279.1069 and 267.1096, respectively) and 106.0535 (no mass-shift) (Figure S6). Furthermore, **M1′** and **M1** shared the same retention time; therefore, **M1′** was further confirmed as a phenol metabolite.

M2′ was eluted at 16.95 min, with a protonated molecular mass of *m/z* 386.1785 (Figure 4). The elemental composition of **M2′** was $^{13}C_2C_{23}H_{23}{}^{15}NNO_2$, indicating that the addition of OCH_2 to **M0′**. The fragment ion of **M2′** at *m/z* 106.0655 was the same as that of **M0′**. The fragment ions at *m/z* 293.1229

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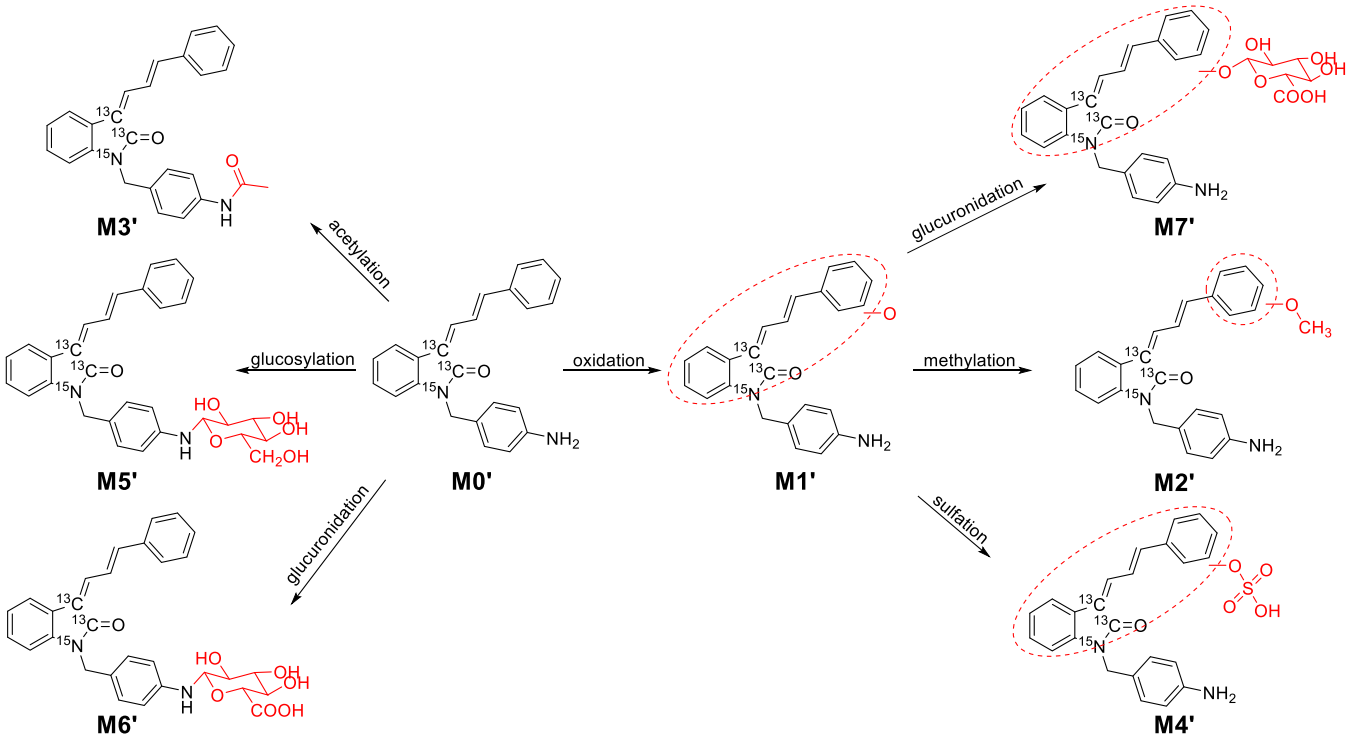

Figure 6. Metabolic pathways of **M0′**.

Table 1. Summarized Information of Metabolites from M0′

metabolite code	metabolic pathways	percent (%)	t_R (min)	molecular formula	exact mass (m/z)	observed mass (m/z)
M0′	parent compound	97.5	17.21	$^{13}C_2C_{22}H_{21}{}^{15}NNO^+$	356.1722	356.1702
M1′	oxidation	0.48	13.17	$^{13}C_2C_{22}H_{21}{}^{15}NNO_2^+$	372.1671	372.1622
M2′	oxidation and methylation	0.50	16.95	$^{13}C_2C_{23}H_{23}{}^{15}NNO_2^+$	386.1827	386.1785
M3′	acetylation	0.09	16.39	$^{13}C_2C_{24}H_{23}{}^{15}NNO_2^+$	398.1827	398.1783
M4′	oxidation and sulfation	0.24	9.71	$^{13}C_2C_{22}H_{21}{}^{15}NNO_5S^+$	452.1239	452.1166
M5′	glucosylation	0.45	12.89	$^{13}C_2C_{28}H_{31}{}^{15}NNO_6^+$	518.2250	518.2186
M6′	glucuronidation	0.60	11.53	$^{13}C_2C_{28}H_{29}{}^{15}NNO_7^+$	532.2043	532.1977
M7′	oxidation and glucuronidation	0.14	7.68	$^{13}C_2C_{28}H_{29}{}^{15}NNO_8^+$	548.1993	548.1919

and 281.1217 were 30 Da bigger than those of **M0′**. The formation of the fragment ion at m/z 145.0658 was attributed to cleavage of the ^{13}C−C double bond; therefore, the formation of **M2′** was attributed to *O*-methylation following the aromatic hydroxylation of **M0′**, and the result was also verified by the detection of **M2** from **M0**. Similarly, **M3′**, **M5′**, and **M6′** were identified as the metabolites formed via *N*-acetylation, *N*-glucosylation, and *N*-glucuronidation of **M0′**, respectively (Figures S7 and S6).

M4′ was eluted at 9.71 min, with a protonated molecular mass of m/z 452.1166 (Figure 5). The elemental composition of **M4′** was $^{13}C_2C_{22}H_{21}{}^{15}NNO_5S$, indicating that the addition of SO_4 to **M0′**. **M4′** was proposed as the sulfate of the hydroxyl **M0′**. The fragment ion of **M4′** at m/z 106.0637 was the same as that of **M0′**. The fragment ion at m/z 279.1069 was 16 Da bigger than that of **M0′**. The fragment ions at m/z 359.0615 and 347.0627 were 96 Da bigger than those of **M0′**, suggesting that **M4′** was not the *N*-sulfate, but the *O*-sulfate of **M0′**. After verification of the detected **M4** having been formed from **M0** (Figure S6), **M4′** was identified as the *O*-sulfate of the hydroxyl **M0′**. Similarly, **M7′** was identified as the *O*-glucuronide of the hydroxyl **M0′** (Figure S7).

Therefore, seven metabolites of **M0′** were identified after the verification of the detected metabolites of **M0**. The proposed metabolic pathways of **M0′** are illustrated in Figure 6 and a summary of information pertaining to those metabolites identified is presented in Table 1. After incubation with human hepatocytes for 3 h at 37 °C, there was approximately 97.5% of the **M0′** detected, demonstrating the metabolic stability of **M0′**. *In vitro*, **M0′** was primarily metabolized by hydroxylation, and secondarily by methylation, acetylation, sulfation, glucosylation, and glucuronidation, etc.

CONCLUSIONS

Compound **M0′** was synthesized and used in the investigation of metabolism in hepatocytes for the first time. The metabolites were studied by UPLC/Q TOF MS, which were verified by unlabeled compound **M0**. Seven metabolites were preliminary discovered and confirmed, including hydroxyl **M0′** (**M1′**), hydroxyl and methylated **M0′** (**M2′**), *N*-acetylated **M0′** (**M3′**), sulfate of hydroxyl **M0′** (**M4′**), the glucose conjugate of **M0′** (**M5′**), glucuronide conjugate of **M0′** (**M6′**), and glucuronide conjugate of hydroxyl **M0′** (**M7′**). After incubation with human hepatocytes for 3 h at 37 °C, compound **M0′** was the main component, accounting for

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97.5% overall, which indicated that compound **M0′** possessed good metabolic stability. This study contributed to establishing an efficient and rapid method for analysis and identification of the metabolites of indolinone analogues *in vitro*, also elucidated the corresponding metabolic pathways. We expected that the discoveries in relation to metabolic pathways and metabolites could provide a certain theoretical basis for drug discovery and pharmacological mechanism research toward the α-syn target and lay a foundation for the study of PET tracers thereon.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.1c00146.

Detailed synthetic steps for compounds **4**−**6** and **M0′**; ^{1}H NMR and HRMS of compounds **4**−**6** and **M0′**; metabolites profiling of **M0** after incubation with human hepatocytes for 3 h at 37 °C by UPLC/Q TOF MS; MS and MS/MS spectra of the metabolites formed from **M0**; MS, MS/MS spectra, and fragmental pathways of the metabolites formed from **M0′**(PDF)

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Notes

The authors declare no competing financial interest.

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