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Untargeted metabolomics analysis assisted by signal selection for comprehensively identifying metabolites of new psychoactive substances: 4-MeO- α -PVP as an example

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Abstract

New psychoactive substances (NPS) have been rapidly emerged as legal alternatives to controlled drugs, which raised severe public health issue. The detection and monitoring of its intake by complete metabolic profiling is an urgent and vital task. Untargeted metabolomics approach has been applied for several NPS metabolites studies. Although the number of such works is relatively limited but with a rapidly growing need. The present study aimed to propose a procedure that includes liquid chromatography high-resolution mass spectrometry (LC-HRMS) analysis and a signal selection software, MetaboFinder, programed as a web tool. The comprehensive metabolites profile of one kind of NPS, 4-methoxy- α -pyrrolidinovalerophenone (4-MeO- α -PVP), was studied by using this workflow. In this study, two different concentrations of 4-MeO- α -PVP along with as blank sample were incubated with human liver S9 fraction for the conversion to their metabolites and followed by LC-MS analysis. After retention time alignment and feature identification, 4640 features were obtained and submitted to statistical analysis for signal selection by using MetaboFinder. A total of 50 features were considered as 4-MeO- α -PVP metabolite candidates showing significant changes ($p < 0.00001$ and fold change >2) between the two investigated groups. Targeted LC-MS/MS analysis was conducted focusing on these significantly expressed features. Assisted by chemical formula determination according to high mass accuracy and *in silico* MS² fragmentation prediction, 19 chemical structure identifications were achieved. Among which, 8 metabolites have been reported derived from 4-MeO- α -PVP in a previous literature while 11 novel 4-MeO- α -PVP metabolites were identified by using our strategy. Further *in vivo* animal experiment confirmed that 18 compounds were 4-MeO- α -PVP metabolites, which demonstrated the feasibility of our strategy for screening the 4-MeO- α -PVP metabolites. We anticipate that this procedure may support and facilitate traditional metabolism studies and potentially being applied for routine NPS metabolites screening.

Keywords: 4-methoxy- α -pyrrolidinovalerophenone (4-MeO- α -PVP), LC-MS/MS, Metabolome, Metabolomics, New psychoactive substances

1. Introduction

Detection of small-molecule drug metabolism is always a high demand for monitoring

drug overdosing or abuse [1]. The use of new psychoactive substances (NPS) continues to emerge in the drug market, which poses an urgent clinical/forensic toxicology and public health issue due to

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the lack of their toxicodynamic and kinetic properties, particularly knowledge of their metabolism [2,3]. NPS include aminoindanes, synthetic cannabinoids, cathinones, ketamine, phenethylamines, tryptamines, piperazines, and other plant-based psychoactive substance [4]. A total of 1124 different NPS have been reported to the United Nations Office on Drugs and Crime's Early Warning Advisory between 2009 and 2021, and the number is growing rapidly [5]. Safety data, carcinogenicity, and toxicology information of most NPS are very limited or even not available. It has been known that synthetic cannabinoids have more serious side-effects than cannabis [6]. Complete metabolic profiling of NPS are crucial for developing detection of intake and link adverse effects to the toxicants. For metabolism studies, since authentic human biosamples are usually not available and the controlled human studies are not practical owing to ethical reasons [2,7], metabolites can be generated through *in vitro* or *in vivo* models which mimic the endogenous enzymatic reaction [8,9].

Evolving techniques such as advanced nuclear magnetic resonance and high-resolution tandem mass spectrometry (HRMS/MS) have improved the capability of metabolites characterization [10,11]. HRMS/MS has great potential to become the gold standard in non-targeted screening [12,13], in which liquid chromatography coupled to mass spectrometry (LC-MS) provides on-line separation and accurate mass measurement to enable the elemental composition determination and confident identification. LC-MS analysis includes more than tens of thousands of features and data processing is one of the key steps to the success of metabolites profiling. One widely-used approach to assist characterizing metabolites includes *in silico* prediction. Based on the prior knowledge, theoretical *m/z* values of predicted metabolites can be calculated according to the formula of the target compound and phase I/II transformation. *In silico* prediction software, such as MetaSite [14], Meteor [15], StarDrop™, SyGMA [16], SOMP [17], and BioTransformer [18] were developed to provide information of sites of metabolism in the early discovery phase. However, the metabolizing enzyme database may not be complete in some software and the prediction was not always accurate for all target compounds [19]. Ellefsen et al. has conducted a suspect screening analysis and reported the metabolites of one kind of NPS, 4-methoxy- α -pyrrolidinovalerophenone (4-MeO- α -PVP), by using human hepatocyte incubation and high-resolution mass spectrometry. Among the 10 identified metabolites, only 3 metabolites matched the results from *in silico* prediction software that

provided 14 candidates, which suggested that software didn't readily predict and has limitations [20]. To this end, several studies have used untargeted metabolomics techniques for NPS metabolite identification [21–27], revealing that traditional metabolism studies of NPS can be supported and facilitated by metabolomics techniques for comprehensive quantitative analysis. Although the number of such works is relatively limited but this field is with a rapidly growing need. To facilitate this application, we aimed to propose an untargeted metabolic profiling workflow, including LC-MS based analysis coupled with a signal selection software programed as a web tool, that has potential to be routinely being applied for NPS metabolites screening. Besides, this approach was demonstrated by comprehensively characterizing the toxicometabolomics of 4-MeO- α -PVP by *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Chemicals and reagents

4-MeO- α -PVP (purity $\geq 98\%$) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Pooled human liver S9 fraction (20 mg protein/mL, from 150 individual donors) was purchased from Corning® UltraPool™ (Woburn, MA, USA). NADPH Regenerating System (Solution A and Solution B) were purchased from Corning® Gentest™ (Woburn, MA, USA). HPLC-grade acetonitrile (ACN, purity $\geq 99.9\%$) was purchased from J.T. Baker (Philipsburg, New Jersey, USA). The internal standard, N-(2-Hydroxybenzoyl)pyrrolidine (N2P), was obtained as powder (purity = 97%) from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Incubation of 4-MeO- α -PVP with human liver S9

The 4-MeO- α -PVP standard was firstly dissolved in the MeOH at 1 mg/mL and followed by being diluted 10 and 200 times the volume with sodium phosphate buffer (50 mM, pH 7.4) to constitute 100 ppm and 5 ppm of 4-MeO- α -PVP solution, respectively. The storage time of buffer-diluted standard solution was no longer than 1 h before enzyme incubation. Each reaction mixture containing 195 μ L sodium phosphate buffer (50 mM, pH 7.4), 25 μ L S9 fraction, and 2.5 μ L Solution B was pre-incubated at 37 °C for approximately 3 min. The reaction was initiated with the addition of 12.5 μ L Solution A and 15 μ L of 100 ppm or 5 ppm 4-MeO- α -PVP solution to make 1 or 20 μ M in the final

mixture. Incubation with five replicates each were performed in a 37 °C water bath for 16 h. The final proportion of MeOH was less than 0.6% in the incubation mixture. Negative controls without 4-MeO- α -PVP were also prepared. Enzyme reactions were terminated by adding 250 μ L cold ACN, stored at –20 °C for 30 min, and each sample was then centrifuged at 20,000 $\times$ g for 20 min at 4 °C. Each vial containing 150 μ L of the resulting supernatants was spiked with 0.02 μ g/50 μ L N2P and subsequently stored at –20 °C prior to analysis.

2.3. *In vivo* animal studies, urine collection, and sample preparation

Male Wistar rats with a 7-week-old weight of approximately 250 g were purchased from Bio-LASCO Taiwan Co. (Taipei, Taiwan). The material of feeding cages was polycarbonate. Breeding environments were maintained on a 12/12 h light/dark cycle (lights off at 20:00 h) at a constant temperature 20 °C and constant relative humidity 60%. Food and water were made available *ad libitum*. One group of male Wistar rats were administered orally with the 4-MeO- α -PVP (40 mg/kg body weight). The 4-MeO- α -PVP samples were dissolved in 1 mL deionized water with the concentration corresponding to rat weight and fed to the rats by oral gavage. The used concentration of 4-MeO- α -PVP ranged from 7.4 to 11.8 mg/mL since the weight of the rat ranged from 185.3 to 295.7 g. The 4-MeO- α -PVP dissolved in water was homogenized by sonication before feeding. Another group administered the deionized water was used as a control group. Six rats were included in each group. After the drug administration, each rat was housed in individual metabolism cages for 24 h. The experimental procedures of the animal experiments were approved by the International Animal Care and Use Committee (NCKU IACUC number: 109360).

Urine samples were collected into glass conical flasks within 24 h and added with sodium azide (0.05% w/v). After collection, the urine samples were aliquoted and stored at –20 °C before analysis. According to the published procedures [28,29], aliquoted urines were centrifuged at 20,000 g at 4 °C for 20 min. To remove glucuronidation and focus on identifying phase I metabolites, the supernatant of the centrifuged urine (400 μ L) was added with 40 μ L β -glucuronidase (6 U) and incubated at 37 °C for 1.5 h. The pH was controlled at 6.0–6.5 as instructed by product document. The deconjugation reaction was terminated by adding 120 μ L of 20% acetic acid. After centrifugation again at 20,000 g for 20 min, supernatants were diluted

with 290 μ L of deionized water contained internal standard N2P and submitted for ultra-high performance liquid chromatography (UHPLC)-HRMS analysis. For each prepared urine sample, the final dilution ratio was three and the concentration of internal standard was 0.05 μ g/ μ L. Aliquot 5 μ L of each prepared urine samples were mixed together as the QC sample for monitoring the instrument variation.

2.4. Liquid chromatography-tandem mass spectrometry (LC-MS) and targeted MS² and MS³ analysis

The prepared samples were analyzed on a Waters ACQUITY UPLC system (Waters, Milford, MA) coupled with an Orbitrap Elite™ Hybrid Ion Trap-Orbitrap Mass Spectrometer system (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was achieved on an ACQUITY UPLC BEH C18 Column (2.1 mm $\times$ 100 mm, 1.7 μ m, Waters, Milford, MA) maintained at 40 °C. The mobile phase consisted of 2% (v/v) ACN with 0.1% (v/v) formic acid in deionized water (A) and 0.1% (v/v) formic acid in methanol (B) at a flow rate of 350 μ L/min with the following linear gradient: 0–2 min, 2% B; 2–16 min, 2–95% B; 16–17 min, 95% B; 17–18 min, 95–2% B; 18–20 min, 2% B.

The mass spectrometer contained a heated electrospray ionization source (HESI). The spray voltage was 3.2 kV, capillary temperature 360 °C, source heater temperature 350 °C, S-lens RF level 60%, sheath gas flow rate 30; auxiliary gas flow rate 15, and sweep gas flow 2 (manufacture's units). Full-scan mass data were acquired in the range of m/z 100–650, with a resolution of 60,000 at m/z 200. AGC target was set at 1e6. As for the targeted MS² analysis, parallel reaction monitoring (PRM) acquisition mode was performed to acquire MS² spectra of all metabolite candidates. The LC condition was the same as mentioned above. The m/z values of the filtered metabolite candidates were set in the global list on the basis of the retention time. Higher energy collision dissociation (HCD)-MS² was used to fragment the targeted ions within a 1-Da isolation window and $\pm$ 4 min RT window from the RT of targeted metabolite candidates. Normalized collision energy (NCE) was set at 35%. AGC target was set at 5e4. For MS³ analysis, specific precursor ions were selected for CID fragmentation with NCE of 35% at MS² and the fragment ions with the identical m/z value of in-source fragment candidates were further selected for HCD-MS³. Parameters of AGC value and resolution were identical to that for the MS² method. Source fragmentation function was

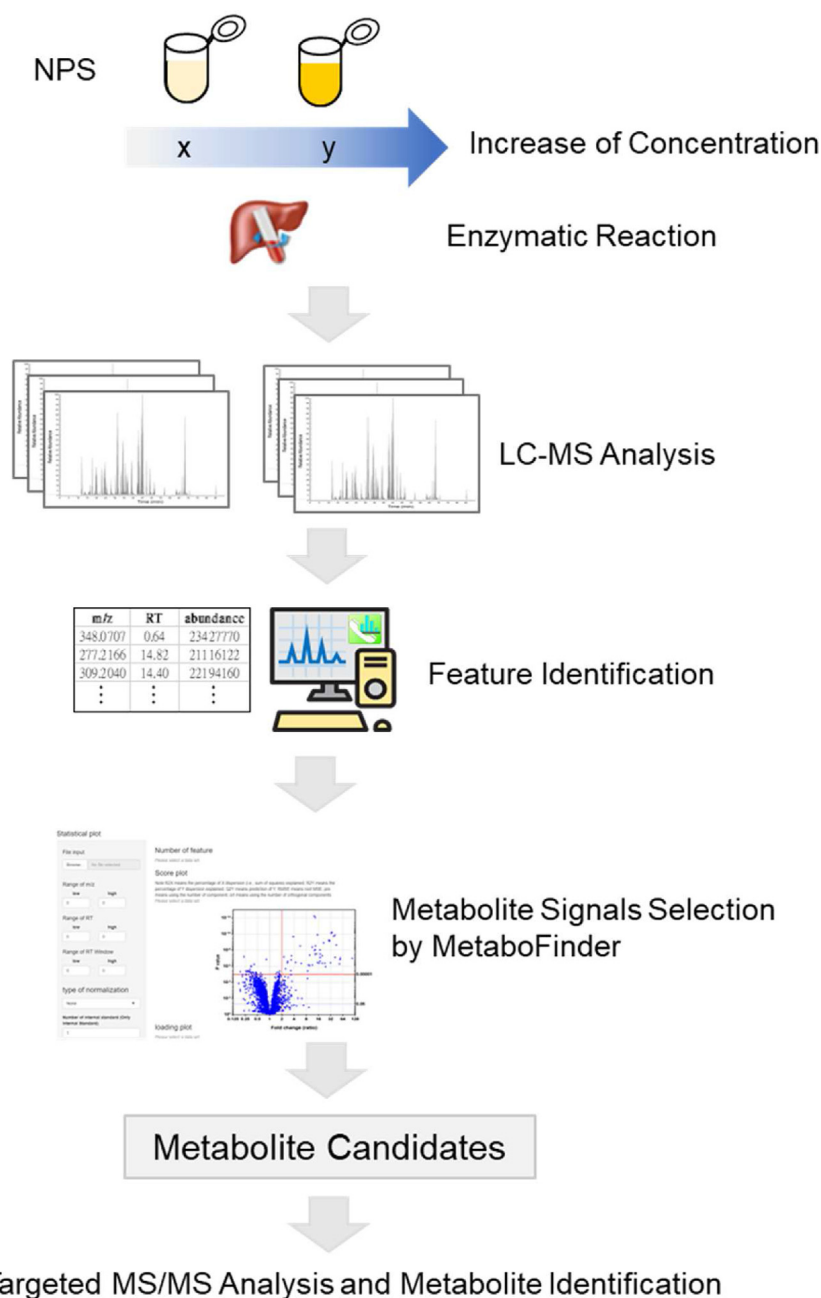


Fig. 1. Analytical approach. One NPS, 4-MeO- α -PVP, with different concentration were incubated with human liver S9 followed by LC-MS analysis. Features in the raw LC-MS files were identified by Progenesis QI software. Matched features were further submitted to statistical analysis by using a web tool, MetaboFinder. Differentially expressed features were filtered out and identified by targeted LC-MS/MS analysis.

enabled and applied with additional collision energy of 0, 5, 20, and 40% for investigating in-source fragments.

2.5. Data processing

All the raw data files from UHPLC-MS analysis were converted to a peak list table, including *m/z* values, retention times (RTs), and signal abundances

by using Progenesis QI software (version 2.0; Non-Linear Dynamics, a Waters company, Newcastle upon Tyne, United Kingdom) for feature detection, alignment, and quantification. Default parameters were used as follows: peak picking limits: automatic; maximum ion charge: 20; quantitation: relative quantitation; isotope number: 0). In order to remove noise and enable enough data points (≥ 10) to be collected for the quantification, custom filtering was

then performed to extract feature signals based on the following parameters: (1) m/z charge state ≤ 2 , (2) peak width > 0.05 min, (3) RT 0–20 min. To adjust differences among each sample, the abundance of individual peaks was subsequently normalized by spiked internal standard, N2P, before the metabolite candidate selection.

2.6. A web tool, MetaboFinder, for signal selection

Selected features were further submitted to an in-house developed software, MetaboFinder, for mining metabolite candidates. MetaboFinder, which can

be accessed through <https://cooperation.shinyapps.io/olsda/>, was programmed as a public web tool. Feature list including feature ID, m/z value, RT, and two groups of sample abundance can be uploaded. Basic instruction was provided in Fig. S1 (<https://www.jfda-online.com/cgi/viewcontent.cgi?filename=0&article=3447&context=journal&type=additional>) while an example of input file can be downloaded from the website. For this study, data obtained from different groups were processed according to setting m/z 0–1000, RT 0–20 min, and RT window 0–0.5 min. Raw abundance was normalized by N2P signal (feature ID: 38) by choosing the “Type of

MetaboFinder

File input

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[Demo dataset.](#)

Range of m/z

low high

0 0

Range of RT

low high

0 0

Range of RT Window

low high

0 0

type of normalization

None

Feature ID of Internal Standard

1

XY-axis of volcano plot

Fold change-p value of t test

Log base of fold change

2

Carry out

Run

Download Table

Download XY axis of score plot

Download XY axis of loading plot

Download XY axis of S plot

Download XY axis of volcano plot

Number of feature

Please select a data set

Score plot

Note: R2X means the percentage of X dispersion (i.e., sum of squares) explained; R2Y means the percentage of Y dispersion explained; Q2Y means prediction of Y; RMSE means root MSE; pre means using the number of component; ort means using the number of orthogonal components

Please select a data set

loading plot

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S plot

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Fig. 2. MetaboFinder. An in-house constructed web tool (<https://cooperation.shinyapps.io/olsda/>) that can process the peak list from LC-MS analysis including m/z , RT, and intensity information. Significantly changed features can be selected out by statistical analysis, visualized by volcano plot, and outputted as a list.

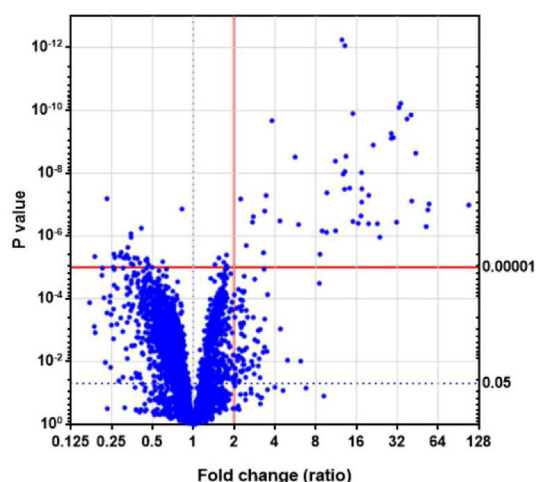


Fig. 3. Low vs high-dose treatments from *in vitro* experiment. Comparing the fold change and *p*-value of individual metabolites. Vertical dashed lines mark the two-fold change and horizontal lines mark the *p*-value cut-off of 0.00001. The upper right quadrants contained significant metabolites upregulated in comparison to low-dose treatment with a *p*-value < 0.00001 and greater than two-fold change.

normalization” as “by internal standard”. After the abundance normalization, *t*-test and fold change calculation were performed in the software to determine the significantly changed features which was visualized by volcano plot and outputted as a list. Parameters used were set as below: XY-axis of volcano plot, fold change-*p* value of *t* test; Log base of fold change, 2. Differentially expressed features found in negative control samples were excluded. It is noted that some real metabolites may not have concentration-dependent difference between low and high concentration samples and will not be sieved out during peak selection process.

2.7. Chemical structure identification

The workflow of identifying metabolite chemical structure was illustrated in Fig. S2 (<https://www.jfda-online.com/cgi/viewcontent.cgi?filename=0&article=3447&context=journal&type=additional>). MS¹ and MS² spectra from targeted analysis with accurate masses (mass error < 5 ppm) of each metabolite candidate were inputted to SIRIUS 4 software version 4.0.1 for generating tentative molecular formula by integrating high-resolution isotope pattern analysis and fragmentation trees [30]. The candidate formula with highest score was considered as the possible molecular formula. Chemical structure of each metabolite formula was inferred from that of parent compound, 4-MeO- α -PVP, based on biotransformation routes. For instance, if a mass difference between one

molecular formula and parent compound equals to one hydroxyl group, this molecular formula was assigned with all possible hydroxylation structures. After compiling a tentative chemical structure list, the Mass Frontier™ software (version 7.0, Waters) was used to generate *in silico* MS² fragment profile of each structure candidate. If the top 5 intense fragments in the experimental MS² spectrum matched to the corresponding *in silico* predicted fragments with mass error < 5 ppm, the inferred structure was considered as the putative chemical structure of the metabolite candidate. In addition to the Mass Frontier, alternative *in silico* MS/MS fragmentation tools such as CSI:FingerID [31] and MS-FINDER [32] can also be implemented in this step.

3. Results and discussion

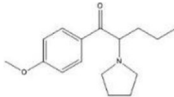
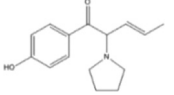
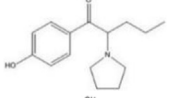
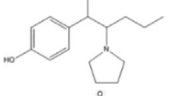
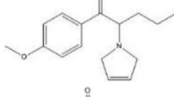
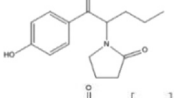
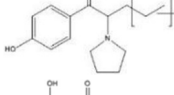
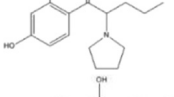
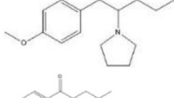
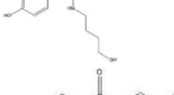
3.1. Workflow for general identification of NPS metabolites

The experimental scheme employed in this study is depicted in Fig. 1. NPS with high or low concentration were both incubated with human liver S9 for the conversion from parent compounds into their metabolites. Samples were analyzed by LC-HRMS. The retention time alignment and feature identification of raw MS data from individual sample was performed. Generated features were further submitted to statistical analysis by using an in-house software, MetaboFinder. Significantly changed features were filtered out and submitted to targeted LC-MS/MS analysis for identification.

3.2. Selection of metabolite candidates

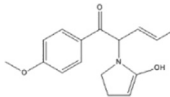
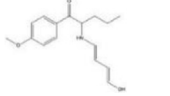
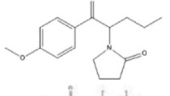
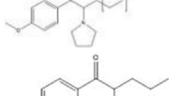
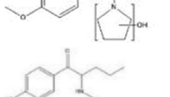
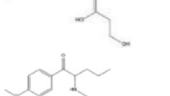
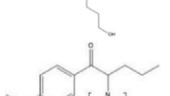
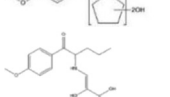
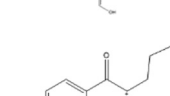
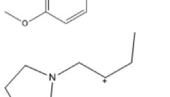
To demonstrate this strategy, 4-MeO- α -PVP, a substituted cathinone, was used as a NPS parent compound. Both 1 μ M and 20 μ M of 4-MeO- α -PVP along with a blank sample (each 5 replicates), spiked with N2P as an internal standard, were incubated with human liver S9 for 16 h and followed by LC-HRMS analysis. To reveal the data points, raw LC-MS data were processed by Progenesis QI to get 22081 features from all samples, and 4640 of which were sieved out based on custom filtering (listed in Table S1 (<https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3447&context=journal&type=additional>)). Selected features were further submitted to an in-house developed software, MetaboFinder, for mining metabolite candidates (Fig. 2). As for 4-MeO- α -PVP, each 5 replicates of 4640 features from 1 μ M to 20 μ M conditions were

Table 1. Characteristics of the 26 identified 4-MeO- α -PVP potential metabolites.

Metabolite ID	Measured <i>m/z</i>	RT (min)	Abundance order ^a	Literature ^b	<i>In vivo</i> Experiment	Fold change	<i>t</i> -test <i>p</i> -value	Chemical information			
								Chemical formula ^c	Theoretical <i>m/z</i> ^d	Mass error (ppm)	Proposed structure
M0	262.1807	8.63		4-MeO- <i>α</i> -PVP	●	33.9	5.87×10^{-11}	C ₁₆ H ₂₃ NO ₂	262.1802	2.05	
M01	246.1494	9.61	12 (1.1%)		●	17.4	8.17×10^{-8}	C ₁₅ H ₁₉ NO ₂	246.1488	2.52	
M02	248.1651	6.71	1 (100%)	M3	●	12.7	1.06×10^{-8}	C ₁₅ H ₂₁ NO ₂	248.1652	2.45	
M03	250.1808	6.18	21 (0.5%)	M2	●	16.4	3.87×10^{-7}	C ₁₅ H ₂₃ NO ₂	250.1802	2.50	
M04	260.1651	11.34	3 (4.0%)	M10	●	13.1	8.58×10^{-13}	C ₁₆ H ₂₁ NO ₂	260.1645	2.22	
M05	262.1444	11.09	11 (1.3%)		●	17.5	3.11×10^{-8}	C ₁₅ H ₁₉ NO ₃	262.1438	2.16	
M06	264.1601	6.38	28 (0.3%)		●	11.2	6.76×10^{-7}	C ₁₅ H ₂₁ NO ₃	264.1594	2.58	
M07	264.1602	5.84	22 (0.5%)		●	8.9	6.83×10^{-7}	C ₁₅ H ₂₁ NO ₃	264.1594	2.91	
M08	264.1965	8.30	4 (3.8%)	M7		28.8	7.67×10^{-10}	C ₁₆ H ₂₅ NO ₂	264.1958	2.53	
M09	266.1754	6.58	15 (0.7%)		●	107.6	1.02×10^{-7}	C ₁₅ H ₂₃ NO ₃	266.1750	1.52	
M10	274.1443	9.79	48 (0.04%)		●	2.7	3.56×10^{-7}	C ₁₆ H ₁₉ NO ₃	274.1438	1.83	

(continued on next page)

Table 1. (continued)

Metabolite ID	Measured m/z	RT (min)	Abundance order ^a	Literature ^b	<i>In vivo</i> Experiment	Fold change	<i>t</i> -test <i>p</i> -value	Chemical information			
								Chemical formula ^c	Theoretical m/z ^d	Mass error (ppm)	Proposed structure
M11	274.1444	10.12	19 (0.6%)		●	3.8	2.09×10^{-10}	C ₁₆ H ₁₉ NO ₃	274.1438	2.23	
M12	276.1599	8.63	5 (2.7%)		●	15.0	3.38×10^{-7}	C ₁₆ H ₂₁ NO ₃	276.1594	1.88	
M13	276.1600	12.35	6 (2.5%)	M11	●	54.8	9.47×10^{-8}	C ₁₆ H ₂₁ NO ₃	276.1594	2.09	
M14	278.1757	6.56	45 (0.1%)		●	31.5	3.57×10^{-7}	C ₁₆ H ₂₃ NO ₃	278.1751	2.04	
M15	278.1757	8.38	8 (2.4%)	M4	●	21.2	1.25×10^{-9}	C ₁₆ H ₂₃ NO ₃	278.1751	2.22	
M16	280.1550	6.56	13 (0.9%)		●	9.6	7.50×10^{-7}	C ₁₅ H ₂₁ NO ₄	280.1543	2.50	
M17	280.1913	8.61	10 (1.4%)	M5	●	23.7	1.07×10^{-6}	C ₁₆ H ₂₅ NO ₃	280.1907	2.03	
M18	294.1706	8.63	2 (26.6%)	M6	●	17.2	2.25×10^{-7}	C ₁₆ H ₂₃ NO ₄	294.1700	1.88	
M19	308.1498	10.96	14 (0.8%)		●	19.6	4.02×10^{-7}	C ₁₆ H ₂₁ NO ₅	308.1492	1.95	
In-source collision derived compounds											
M0'	191.1071	8.63	7 (2.5%)	—	—	15.0	1.25×10^{-10}	C ₁₂ H ₁₅ O ₂ ⁺	191.1067*	2.09	
M02'A	126.1280	6.71	31 (0.2%)	—	—	13.4	2.87×10^{-9}	C ₈ H ₁₆ N ⁺	126.1278*	1.59	

M02/B	177.0914	6.71	26 (0.4%)	—	—	5.6	3.01×10^{-9}	$C_{11}H_{13}O_2^+$	177.0911*	1.69	
M03'	232.1702	6.18	27 (0.3%)	—	—	4.3	3.26×10^{-7}	$C_{15}H_{22}NO^+$	232.1696*	2.58	
M04'	175.1121	11.34	33 (0.2%)	—	—	2.2	6.53×10^{-8}	$C_{12}H_{15}O^+$	175.1118*	1.71	
M08'	246.1859	8.30	9 (2.3%)	—	—	29.9	7.23×10^{-10}	$C_{16}H_{24}NO^+$	246.1853*	2.44	

^a Abundance ratio: abundance/max abundance (M02, m/z : 248.1651, RT: 6.71 min).

^b 4-MeO- α -PVP metabolite identifier reported in the literature [20].

^c Calculated by SIRIUS 4 software using MS¹ and MS² spectra.

^d The theoretical m/z for all the ions are $[M+H]^+$, except the ion denoted with a start means the ion is $[M]^+$.

processed by setting m/z 0–1000, RT 0–20 min, RT window 0–0.5 min and normalized by N2P signal (m/z 192.1024) abundance. After the abundance normalization, t -test p value and fold change of each normalized feature were calculated and illustrated as volcano plot (Fig. 3). As depicted in the volcano plot, 50 features, located in the upper-right panel, were considered as 4-MeO- α -PVP metabolite candidates for they show significant change ($p < 0.00001$ and fold change >2) between two different concentration of parent compound (listed in Table S2 (<https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3447&context=journal&type=additional>)). The significance level ($p < 0.00001$) used in our study has been adjusted by dividing 0.05 with feature number (~5000) according to Bonferroni procedure [33,34]. Fold change greater than 2 was used based on reference [35]. It is noted that some real metabolites might not have concentration-dependent difference between low and high concentration samples and will not be sieved out during peak selection process.

3.3. Targeted LC-MS/MS analysis and chemical structure elucidation for metabolite identifications

Targeted LC-MS/MS analysis were performed on the 50 selected potential metabolites. Although MS² spectra were collected for the selected candidates, no spectral or structural database was available for most NPS metabolites, which hampered the metabolites identification. To facilitate the process, molecular formula for each feature was obtained based on measured m/z value and MS² spectrum by using SIRIUS 4 and that with the highest score was accepted [30]. The potential chemical structures were manually calculated based on biotransformation rules for each formula, which was also inputted to Mass Frontier software for generating *in silico* MS² fragments. A total of 26 metabolites (M01~M19, M0', M02'A, M02'B, M03', M04', and M08'), as listed in Table 1, were identified. Take M02 as an example, M02 was identified from feature 17. Its extracted ion chromatogram (EIC) showed the retention time at 6.7 min and measured m/z value at 248.1651 (Fig. 4A). The molecular formula of M02 peak is proposed as $C_{15}H_{21}NO_2$ by SIRIUS 4 software while chemical structure was manually calculated as 4-hydroxy- α -pyrrolidinovallero-phenone (4-OH- α -PVP) based on biotransformation routes, which has the mass error of 2.45 ppm compared to the theoretical value. It was proposed that 4-OH- α -PVP derived from the *O*-demethylation of the 4-methoxy moiety in the aromatic ring of 4-MeO- α -PVP. As depicted in Fig. 4B,

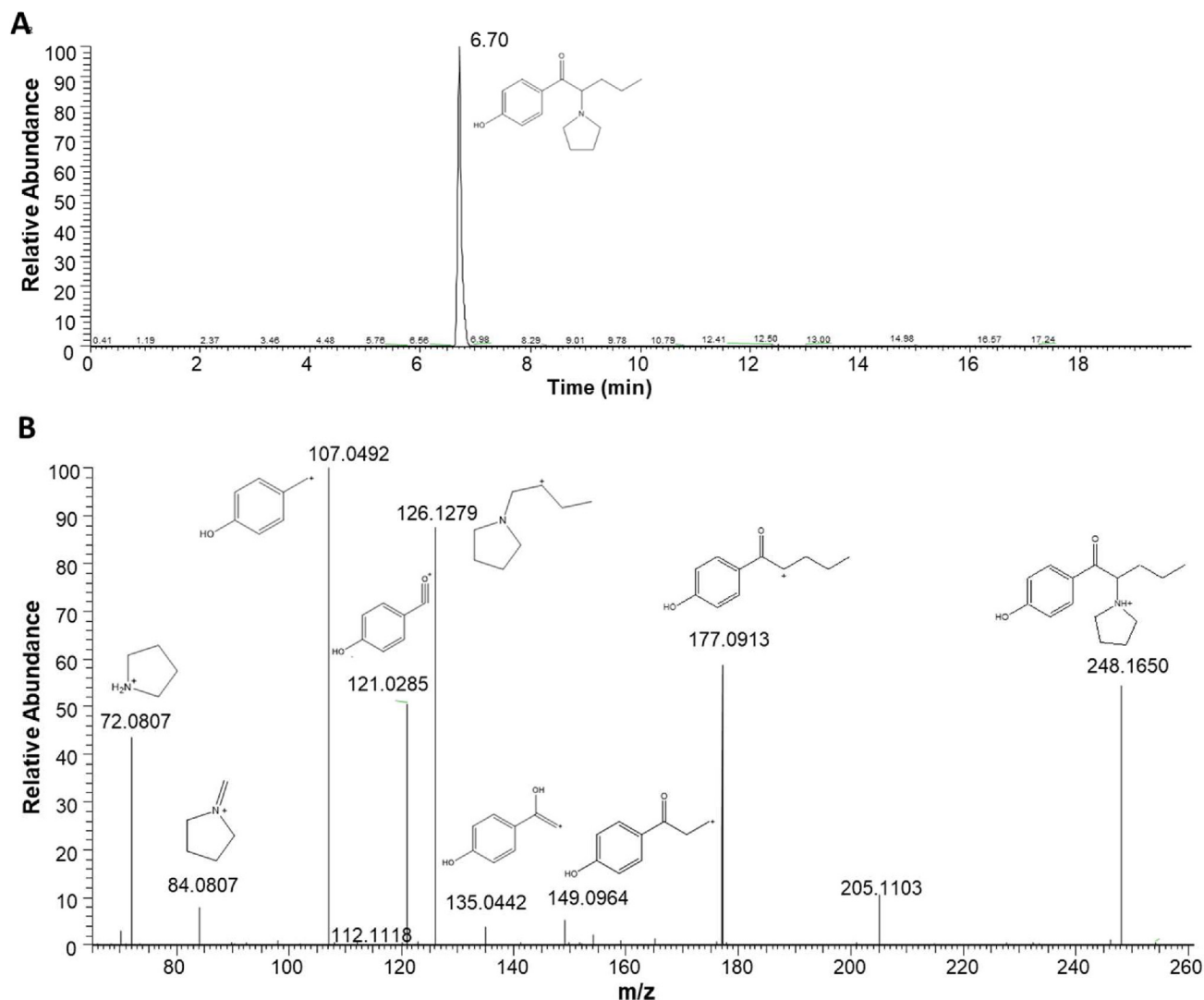


Fig. 4. EIC and MS² spectra of M02. (A) M02 were observed at 6.7 min with *m/z* value at 248.1651. (B) Fragments at *m/z* 121.0285, 126.1279, 135.0442, and 177.0913 confirmed the identification as 4-OH- α -PVP.

MS² spectrum containing fragments at *m/z* 126.1279, 121.0285, 135.0442, and 177.0913 confirmed the identification. The mass errors of product ions between experimental data and Mass FrontierTM predication data were all below 3 ppm. The metabolite, 4-OH- α -PVP, has been reported as the dominant metabolite derived from incubation of 4-MeO- α -PVP with human liver microsomes and human hepatocyte [20].

Among the 26 identified chemical structure, M0', M02'A, M02'B, M03', M04', and M08' were considered to be derived from their precursor ions, M0, M02~04, and M08, by in-source fragmentation, meeting the following three conditions: (1) the suspected in-source fragment coeluted with its precursor ion; (2) in-source fragmentation ion must exist in the MS² spectrum of precursor ion; (3) MS² spectrum of the in-source fragmentation ion was

consistent with the MS³ spectrum from the precursor ion. Take M02 and M02'B as examples. M02 (*m/z* 248.1651, singly charged) and M02'B (*m/z* 177.0914, singly charged) both eluted at 6.7 min and were identified as 4-OH- α -PVP and 1-(4-hydroxyphenyl)-1-oxopent-2-ylum, respectively (Fig. 5A and B). The structure of M02'B was a loss of pyrrolidine from M02. Besides, M02'B was observed in the MS² spectrum of M02. The MS³ scan of *m/z* 177.0914 which was one of the product ion of M02 (Fig. 5C) showed similar pattern with product ion pattern from M02'B in full scan data (Fig. 5D). We further changed the collision energy of in-source fragmentation and found that as the collision energy increased, the abundance of M02'B that was produced from M02 increased (Fig. 5E). An in-source collision fragment identified is a proof that the precursor compound of which did present

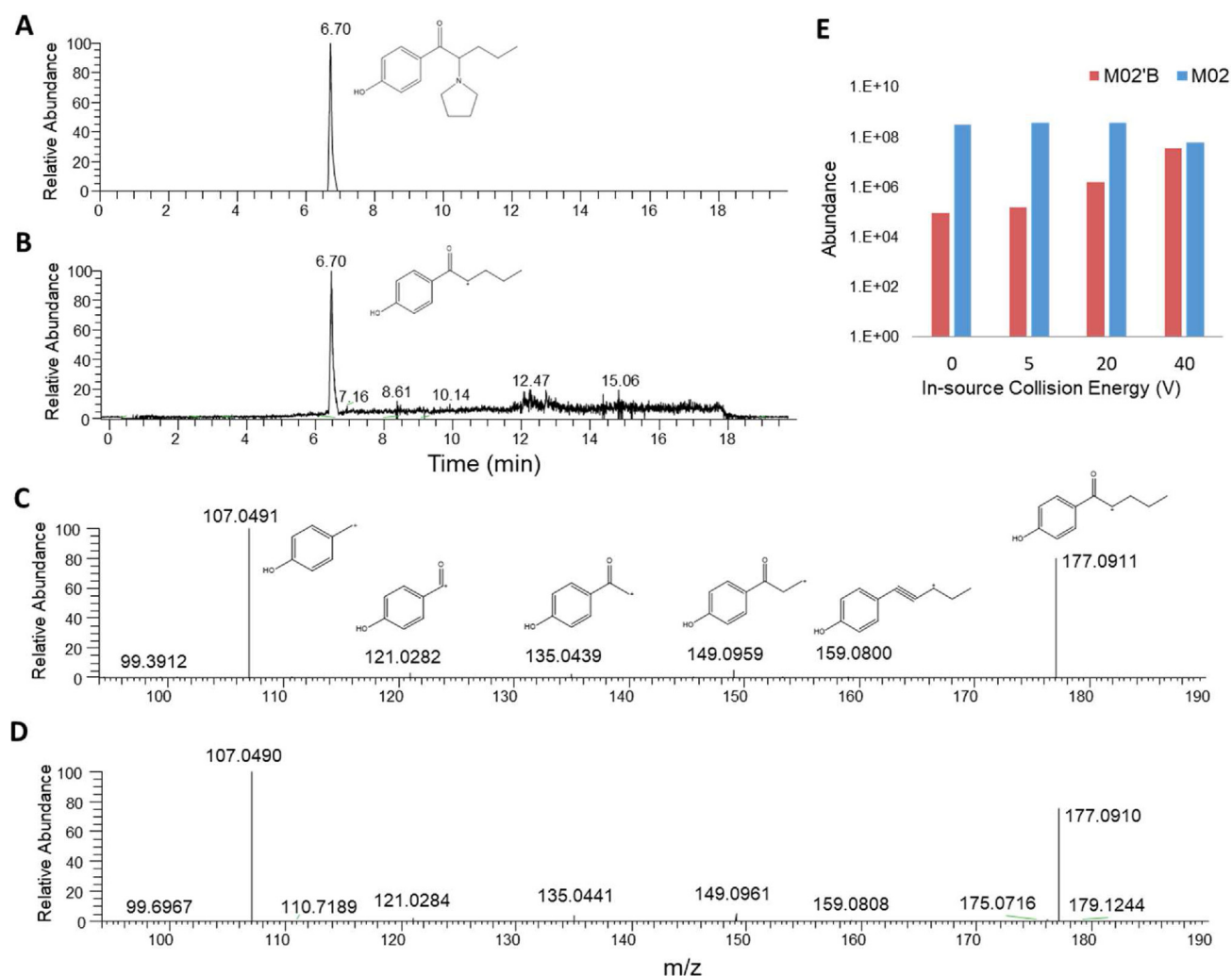


Fig. 5. Metabolites derived from in-source fragmentation. (A) EIC of M02 (m/z 248.1651, singly charged) and (B) its in-source fragment, M02'B (m/z 177.0914, singly charged). (C) The MS^3 scan of one product ion (m/z 177.0914) of M02. (D) Product ion pattern from M02'B. (E) Abundance of M02 and M02'B under different collision energy of in-source fragmentation.

in the MS dataset. Here, a total of 19 metabolites were putatively identified as 4-MeO- α -PVP metabolites.

Ten 4-MeO- α -PVP metabolites identified with full scan and MS^2 spectra has been reported in Ellefsen et al.'s work [20]. It is noted that all the reported 4-MeO- α -PVP metabolites in Ellefsen et al.'s work was also found in our study, with the exception of two that yielded from O-demethylation and N-dealkylation of the pyrrolidine ring of M02 ($C_{11}H_{15}NO_2$, m/z 194.1182) and aliphatic N-oxidation ($C_{16}H_{23}NO_3$, m/z 278.1749). We speculated that this metabolite may be generated specifically from incubation with human hepatocytes. The metabolic pathway of 19 identified 4-MeO- α -PVP metabolites were illustrated in Fig. 6. Eight metabolites that has been reported in the previous

literature were presented with solid-line arrow and 2 metabolites presented with dash line arrows indicated that it has been reported but was not found in our study. Eleven novel 4-MeO- α -PVP metabolites identified by using our strategy were shown with red arrow. For these newly identified targets, M01 and M07 derived from M02 through dehydrogenation and hydroxylation, respectively. M14 was the hydroxylated metabolite of parent compound and M06 was the O-demethylated form of M14. M13 was the results of carbonylation of the pyrrolidine ring, which is a common biotransformation for α -pyrrolidinophenones [36]. We detected that it further converted to a O-demethylated form, M05. Another pyrrolidine ring opening and hydroxylated metabolite (M17) generated M09, M12, and M19 through O-

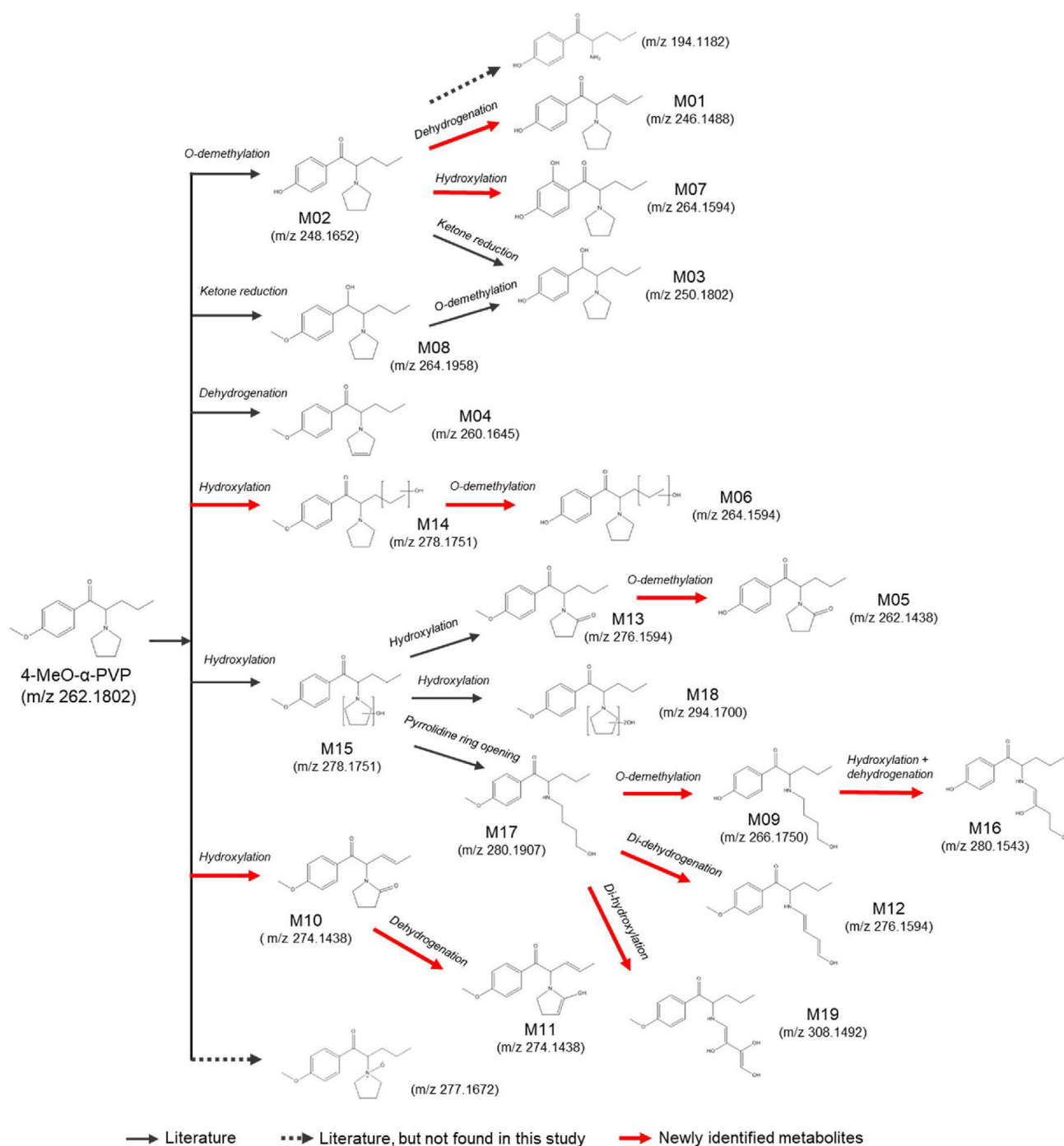


Fig. 6. Proposed metabolic pathway of 19 identified 4-MeO- α -PVP metabolites. Every metabolite is labeled by its metabolite identifier as listed in Table 1 and theoretical m/z value. Metabolites with black arrows were reported in the literature [20]; dotted arrows refer to metabolites that were reported in the literature but were not found in our study; red arrows represent newly identified metabolites of 4-MeO- α -PVP. Metabolic reactions were annotated on the arrows.

demethylation, di-dehydrogenation, and di-hydroxylation reactions, respectively. M16 was the hydroxylated plus dehydrogenated form of M09. M10 was another hydroxylated metabolite of the parent compound which was converted to

M11 through dehydrogenation reaction. The metabolites at m/z 194.1182 and 277.1672 were not found in our strategy, which may suggest that they might be specific metabolites from hepatocyte incubation.

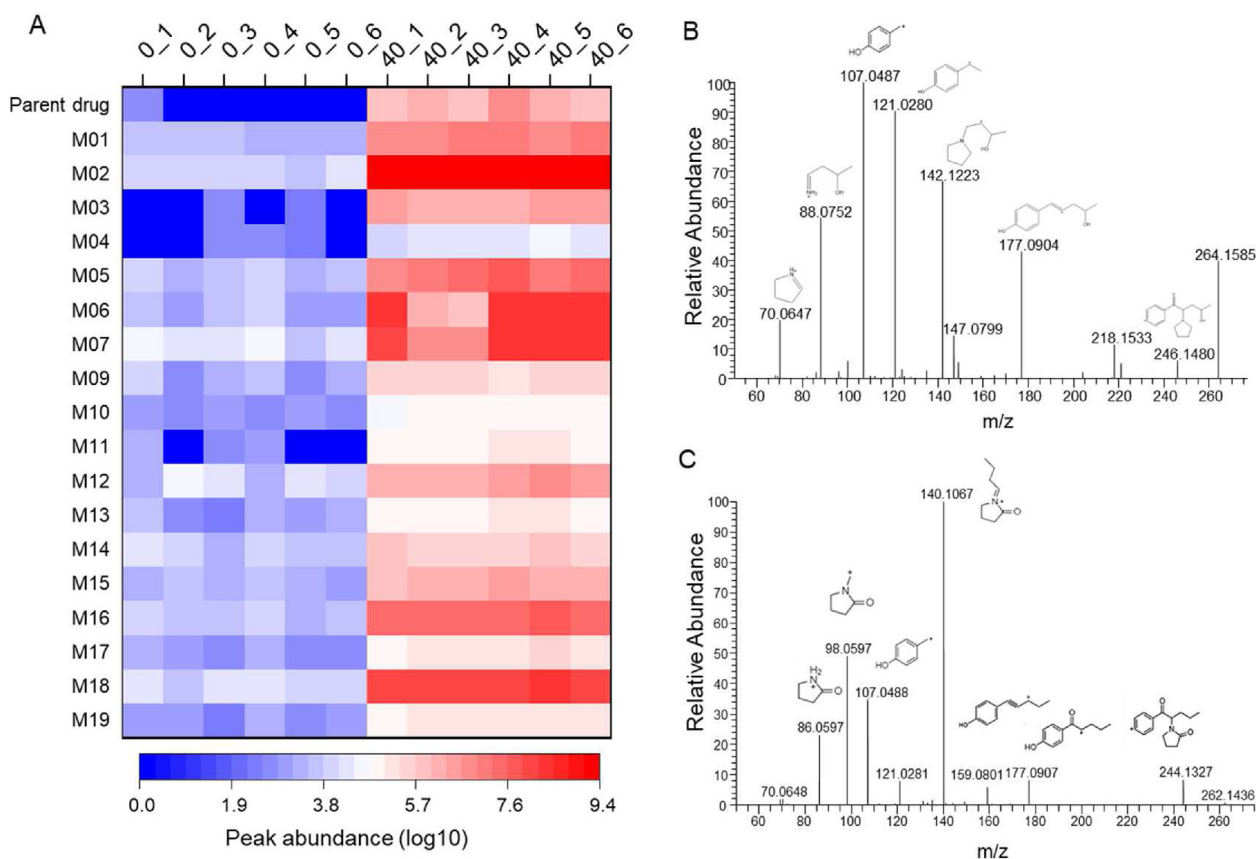


Fig. 7. Confirmation of 18 metabolites in rat urine. (A) Heatmap of the log₁₀ abundance of parent drug and 18 metabolites from rats being administered orally with 40 or 0 mg/kg of 4-MeO- α -PVP. MS² spectra of (B) M05 and (C) M07.

3.4. In vivo confirmation of identified metabolites

It is important to note that although incubation with enzymes reflects liver metabolism, additional processes were also observed in organism, such as extrahepatic metabolism and enterohepatic circulation existed. Since particular enzyme metabolism only represent one aspect of drug elimination, it is vital to confirm the identified metabolites in *in vivo* specimens. To highlight the capability of our strategy, the presence of the 19 metabolites derived from human liver S9 fraction incubation was confirmed in rat urine specimens. Six rats for each group were administered orally 0 or 40 mg/kg body weight of 4-MeO- α -PVP and their urines were collected after 24 h. After sample preparation, each sample was submitted to targeted MS² analysis focused on the 19 identified metabolites. The presence of 18 metabolites were confirmed in the rat urine with both MS¹ and MS² analyses (as annotated in Table 1). All of them showed higher abundance in high dose compared to zero dose of intake (Fig. 7A). Take two

newly discovered 4-MeO- α -PVP metabolites, M05 and M07, as examples. M05 contained characteristic fragments, *m/z* 86.0597, 98.0597, 107.0488, 140.1067, 159.0801, 177.0907, 244.1327 that derived from the structural information of parent compound, the carbonylation of the pyrrolidine, and O-demethylation of the 4-methoxy moiety (Fig. 7B). In Fig. 7C, characteristic fragments, *m/z* 70.0647, 88.0752, 107.0487, 121.0280, 142.1223, 177.0904, 246.1585 illustrated that M07 contained the structure of parent compound and the O-demethylation of the 4-methoxy moiety followed by introduction of the second hydroxyl group at the para-position. MS² spectra that were obtained from the *in vivo* animal experiment were identical to that from the *in vitro* experiments. The EIC (from both high and zero doses) and MS² spectra of the other 16 metabolites were provided in Supporting Fig. S3 (<https://www.jfda-online.com/cgi/viewcontent.cgi?filename=0&article=3447&context=journal&type=additional>). It is speculated that M08 was not detected in the rat urine may be due to its tendency

to transform into M03. The presence of 18 metabolites obtained from *in vitro* experiment confirmed the results from *in vitro* experiment.

4. Conclusions

Lots of works need to be conducted to comprehensively identify metabolomics profiles for certain NPS. Here, we presented a workflow coupling LC-MS analysis, data processing by in-house statistical tool, and structure identification, to assist traditional metabolism studies, which may be applied for routine characterization of metabolites derived from NPS or other drugs. The MetaboFinder software was designed for the one-step drug metabolite candidate discovery. Users can do the *m/z* and retention time-based feature filtering, automatic data normalization, and univariate/multivariate statistical analysis all in this software, which may facilitate the metabolites profiling process. The feasibility of this workflow has been demonstrated for comprehensively characterizing 4-MeO- α -PVP metabolites. A total of 11 novel metabolites of 4-MeO- α -PVP were putatively characterized by *in vitro* and *in vivo* experiment. In general, there was a good agreement between them. A limitation of *in vivo* experiment conducted here was that it was based on an animal model. Confirmation in real samples from human consumers is required for further use of these metabolites. In principle, this procedure can be also applied to analyze metabolites of other NPS. We believe that aside from developing comprehensive knowledge to metabolomes, advances and ease of use in the analytical workflow can eliminate the loading and time for evaluation of NPS, which could facilitate the establishment of monitoring system for public health.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

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