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Targeted Data-Independent Acquisition and Mining Strategy for Trace Drug Metabolite Identification Using Liquid Chromatography Coupled with Tandem Mass Spectrometry

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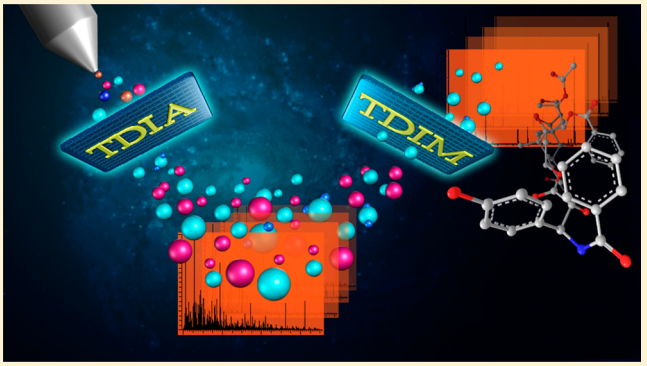
Supporting Information

ABSTRACT: Detection and identification of unknown or low-level drug-related metabolites in complex biological materials is an ongoing challenge. A highly selective and sensitive method could be a possible solution. Here, we proposed a targeted data-independent acquisition and mining (TDIAM) strategy for the rapid identification of trace drug metabolites using ultra-high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HRMS/MS). In this strategy, raw data is acquired by a novel tm-MS scan, which contains an interleaved full MS scan with a targeted mass range and a product ion scan by selecting all ions in the targeted mass range as precursor ions. For efficient discovery of metabolites, raw data are analyzed by a new postacquisition processing method, Molecule- and Fragmentation-driven Mass Defect Filters (MF-MDFs), which was developed based on the fragmentation of parent drug to pick out molecular ions and fragment ions of potential metabolites from the complex matrix. When applying the proposed strategy to paclitaxel metabolism research, we successfully identified 10 metabolites, among which six were not previously reported. The results demonstrated that TDIAM greatly improved throughput, detective sensitivity, and selectivity and, more importantly, yielded almost the same spectrum as traditional HRMS/MS. Therefore, TDIAM provides structure-enriched evidence to confirm the existence and elucidate the structures of metabolites. This strategy is suitable for identification of metabolites present at low concentrations in a complex matrix, and it has the potential to provide an efficient, sensitive, and labor-saving solution for drug metabolite research.



As an integral part of the early stage of drug discovery, drug metabolism research provides a wealth of information related to formation of active metabolites, the route and rate of drug clearance from the body, side effects, and formation of reactive or other toxic metabolites.[1−3] However, it is time-consuming to detect and identify drug metabolites: *in vivo* samples are very complex, and they contain many endogenous components, whereas drug-related metabolites are usually present at much lower concentrations; consequently, the metabolites are often not clearly visible in the total ion current chromatogram.[4] Metabolite identification studies are also labor-intensive, because most current strategies for drug metabolism research are not selective enough to acquire purified metabolite-related data and yield structural information on the putative position of the particular biotransformation of interest.

Because LC-MS is one of the leading techniques used in drug metabolism research, due to its high sensitivity and capacity for structure elucidation,[5−13] several scan functions, such as IDA,[14−16] selected reaction monitoring (SRM),[17] and precursor and neutral loss scan based on triple quadrupole instruments,[18] have their own characteristics and are extensively used in drug metabolism studies. However, these scan functions are relatively insensitive, have a long duty cycle, and require multiple injections to monitor multiple precursors and neutral losses in order to be comprehensive.[19] Recently developed data-independent acquisition (DIA) derived methods,[20−25] such as MS^E, SWATH, and MSX, should be more suitable for systematic and comprehensive screening, allowing for data collection of intact ions and fragment ion data by two interleaved scan functions using low and high collision energy, respectively. Although these currently used methods for

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metabolite research can efficiently collect rich information, the complex matrix of biological samples is the main obstacle to applying them to *in vivo* studies. It is difficult to discover low-concentration metabolites due to interference from abundant endogenous components, which may also increase the false-positive rate.

Among several data-processing methods used for the rapid detection of metabolites in complex biological matrixes, such as software-assisted library searching,[26] high-resolution-extracted ion chromatography (HR-XIC),[27] isotope pattern filter (IPF),[28] and background subtraction (BS),[29] mass defect filter (MDF)[30] has become an attractive option. In this method, metabolite ions are segregated from interference ions in a full-scan MS data set by imposition of preset criteria around the mass defects of the parent drug and selected core substructures. However, the main limitation of MDF is that MDF-processed ion chromatograms often display more false-positive peaks and cannot provide sufficient structural information about metabolites. Therefore, the major challenge of in vivo drug metabolism research is to establish a targeted strategy for detecting and identifying unknown or low-level drug-related metabolites in a large excess of biological material.

Paclitaxel is a taxane diterpene amide, which was first extracted from the stem bark of the western yew, and it has proven to be useful in the treatment of a variety of human neoplastic disorders, including platinum-resistant ovarian cancer, breast and nonsmall cell lung cancer, and leukemia.[31,32] Pharmacokinetic studies[33−35] in humans, rats, and mice showed that, after i.v. administration, paclitaxel is metabolized to differentiated metabolites that have marked species differences but are not glucuronated or sulfated.

In this study, we established a novel targeted data-independent acquisition and mining (TDIAM) strategy for *in vivo* metabolite identification (Figure 1), based on ultra high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC-HRMS/MS). The targeted data-independent acquisition (TDIA) collects intact ions and fragment ions in a wide Q1 ion pass window predicted by drug metabolism rules, with the aim of effectively minimizing interference of abundant endogenous components and significantly increasing sensitivity and selectivity for metabolite detection. In order to exploit the advantages of TDIA to the full, we set up a novel targeted data-independent mining (TDIM) approach, Molecule- and Fragmentation-driven Mass Defect Filters (MF-MDFs), whose development was based on the fragmentation of parent drug.

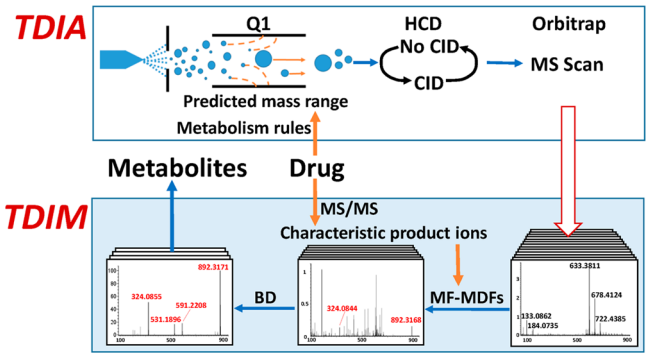

Figure 1. Schematic diagram of TDIAM. BD: Blind Deconvolution; MF-MDFs: Molecule- and Fragmentation-driven Mass Defect Filters.

EXPERIMENTAL SECTION

Data Acquisition with TDIA. High-resolution MS and MS/MS spectra were acquired in positive mode on the basis of our proposed TDIA method by the implementation of a novel targeted multiplex MS (tm-MS) scan, which contains two interleaved scan experiments, full MS scan and product ion (PI) scan. The full MS scan was performed with a targeted mass range from m/z 600 to m/z 1300 without collision induced dissociation (CID), and the PI scan was performed with a wide scan range (m/z 100−1300) in orbitrap to obtain product ions originated in the targeted Q1 mass range (m/z 600−1300) of precursor ions using CID at a high normalized collision energy (NCE) (18%, for paclitaxel metabolism research).

Data Processing with MF-MDFs Coupled with Blind Deconvolution. The Xcalibur Qual browser version 3.0 software (ThermoFisher Scientific) was used to convert the data format of the tm-MS spectrum (spectrum acquired by tm-MS scan) from *.raw (after subtracting background) to *.cdf. Then the *.cdf data was processed with MF-MDFs coupled with blind deconvolution (BD) using home-modified ChemPattern software (Chemmind Technologies Co., Ltd.). For MF-MDFs, traditional MS/MS analysis of paclitaxel was performed (NCE, 18%) to acquire the characteristic product ions. Then the accurate masses of the parent drug and its fragment ions were served as templates, and a window of ±50 mDa was defined from the mass defect of the parent drug and its fragment ions. For paclitaxel metabolite research, six templates were used for MF-MDFs (Table S1, Supporting Information). Next, the filtered spectrum was processed with a blind deconvolution using the following parameters: peak combination threshold, 0.93; minimal peak area (%), 0.01; ion peak tolerance, 0.05; deconvolution S/N, 3; baseline determination threshold, 0.9; addition and isotope ion combination, off; total fragment ions detection, on. More details on deconvolution are provided in METHODS, Supporting Information.

Validation. Traditional MS/MS scan aimed at M1−M10 was performed with an isolation window of 2 Da (NCE at 18%). We compared the tm-MS spectra of M1−M10 obtained by TDIAM with the HRMS/MS spectra of M1−M10 acquired by a conventional MS/MS scan to validate the results.

RESULTS AND DISCUSSION

Because there is a structural correlation between a drug and its metabolites, the mass range of potential metabolites can be predicted according to the general rules of drug metabolism.[30,36] Moreover, in mass spectrometry analysis, characteristic PIs of a drug can indicate the existence of metabolites and contribute to identification of candidate metabolites' structures.[25] Our TDIAM strategy was proposed based on this structural correlation between a drug and its metabolites, and it aims not only to simultaneously acquire comprehensive and targeted information for metabolite identification, but also to effectively minimize interference of abundant endogenous components in order to significantly increase the sensitivity and selectivity of metabolite detection.

TDIA was performed by implementing a novel targeted multiplex MS (tm-MS) scan. In the tm-MS scan, ions in the predicted mass range selected by Q1 were analyzed in two interleaved scan experiments: full MS scan and PI scan. The full MS scan was performed with a targeted mass range, whereas the PI scan was performed with a wide scan range to obtain

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product ions originating in the targeted mass range of precursor ions using a high NCE.

TDIA for Comprehensive Metabolite Detection. To evaluate the performance of TDIA for metabolite detection, we used the signal-to-noise (S/N) ratio of compound M9, a previously unreported metabolite of paclitaxel, to compare the sensitivity of TDIA versus DIA. Figure 2 shows that the S/N

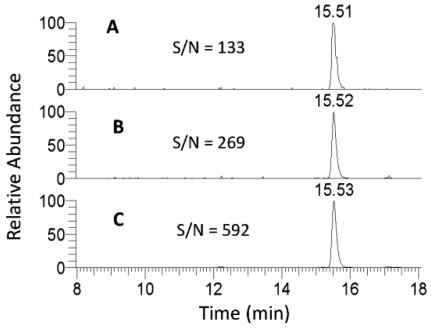

Figure 2. XICs of M9 (m/z 874.3045) acquired by tm-MS scan mode with three different scan range widths. (A) scan range, m/z 100−1500; (B) scan range, m/z 600−1300; (C) scan range, m/z 872.5−875.8.

ratio was greatly elevated, while the scan range was narrowed, and the peak shape was ameliorated when using a narrow scan range. This was because the quadrupole mass filter was set to eject all masses outside the selected mass range and could selectively accumulate ions in the preset mass range. Thus, the tm-MS scan in TDIA exhibited superior acquisition of targeted and comprehensive information on potential metabolites, high sensitivity, and minimal interference from abundant endogenous components in biological materials. By predicting the mass range of potential metabolites of paclitaxel according to the general rules of drug metabolism, a targeted scan range of m/z = 600−1300 was determined to be optimal for our proposed TDIAM strategy.

The S/N ratio was also significantly influenced by the automatic gain control (AGC) target value. Figure 3 shows that

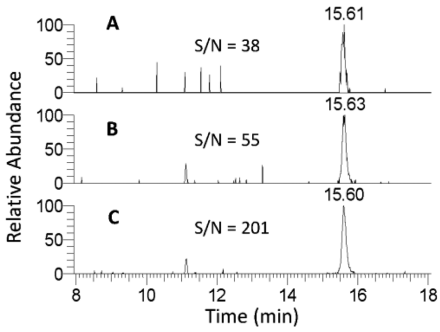

Figure 3. XICs of M9 (m/z 874.3045) acquired when setting three different AGC target values. (A) AGC target, $1e^5$; (B) AGC target, $1e^6$; (C) AGC target, $5e^6$.

the S/N ratio of M9 was higher and the peak shape was improved when the AGC target value was increased. In this experiment, the maximum target capacity of the C-trap (AGC target) was defined as $1e^5$, $1e^6$, or $5e^6$, and the ion injection time was varied to allow the accumulation of a predefined number of ions (AGC target) in the Orbitrap mass analyzer. A previous study[37] suggested that the total detectable ion population in the trap is defined by AGC; therefore, increasing the AGC target value is an effective approach to improving the detectability of the ion of interest in a complex mixture, leading to an improved S/N and lower detection limits. Figures 2 and 3 illustrate another issue deserving attention: the peak was pronged, and some shoulder peaks occurred when the AGC target value was too low or the scan range was set too wide, which may not only have an adverse effect on peak shape but may ultimately lead to false negative results in drug metabolism studies.

TDIM for Rapid Metabolite Discovery. Although TDIA could effectively improve the specificity of the data acquisition methods used in metabolite research, it is still difficult for researchers to quickly identify molecular peaks of potential metabolites from raw tm-MS data. As reported previously,[30] the Mass defect filter (MDF) technique was used to enable high-resolution mass spectrometers to be utilized for detection of both common and uncommon drug metabolites. This technique works by segregating metabolite ions from interference ions in a full-scan MS data set by imposing preset criteria around the mass defects of the parent drug and selected core substructures. In this study, based on the fragmentation of a parent drug, we developed a Molecule- and Fragmentation-driven Mass Defect Filters (MF-MDFs) method by simultaneously picking out molecular ions and characteristic fragment ions related to potential metabolites in a targeted mass defect range.

MF-MDFs and blind deconvolution were used as TDIM to process the raw tm-MS data for rapid discovery of metabolites from a complex matrix. The results are shown in Figure 4. M1 ($[M + Na]^+$, m/z 892.3156) was used to illustrate the capability of MF-MDFs to purify the tm-MS spectra. In contrast to Figure 4 (A2), in which both the molecular ion and fragment ions were hidden in the mixed raw tm-MS spectra, the molecular ion at m/z 892.3179 was visible in Figure 4 (B2) after processing by MDF. However, there were still many other possible molecular ions in the processed spectrum, slowing metabolite identification. By contrast, in Figure 4 (C2), the tm-MS spectrum processed by MF-MDFs retained both the molecular ion of m/z 892.3171 and fragment ions of m/z 324.0855, m/z 531.1896, and m/z 591.2208; therefore, the existence of M1 was further confirmed due to the observation of characteristic fragment ions. With chemometric analysis, blind deconvolution was chosen to complement MF-MDFs to remove more unrelated ions. As further processed with blind deconvolution, the tm-MS spectrum (Figure 4 D2) was similar to the HRMS/MS spectrum (Figure 4 E) acquired by a traditional MS/MS scan, demonstrating the superior performance of our proposed MF-MDFs coupled with a blind deconvolution method. Other tm-MS spectra of M2-M10 processed by TDIM are shown in Figure S1, Supporting Information.

Unlike MDF, which only picks out molecular ions of potential metabolites, MF-MDFs filtered out the majority of the background ions in tm-MS spectra while preserving molecular ions and fragment ions related to potential metabolites. Thus, this method not only confirmed the existence of metabolites but also yielded structural information regarding the putative position of the particular biotransformation of interest. Thus, TDIM based on MF-MDFs coupled with blind deconvolution represents a powerful tool for dealing with data acquired by TDIA for rapid identification of metabolites.

Application of the TDIAM Strategy to Paclitaxel Metabolite Research. Using the TDIAM strategy, we successfully detected paclitaxel and 10 metabolites in mouse

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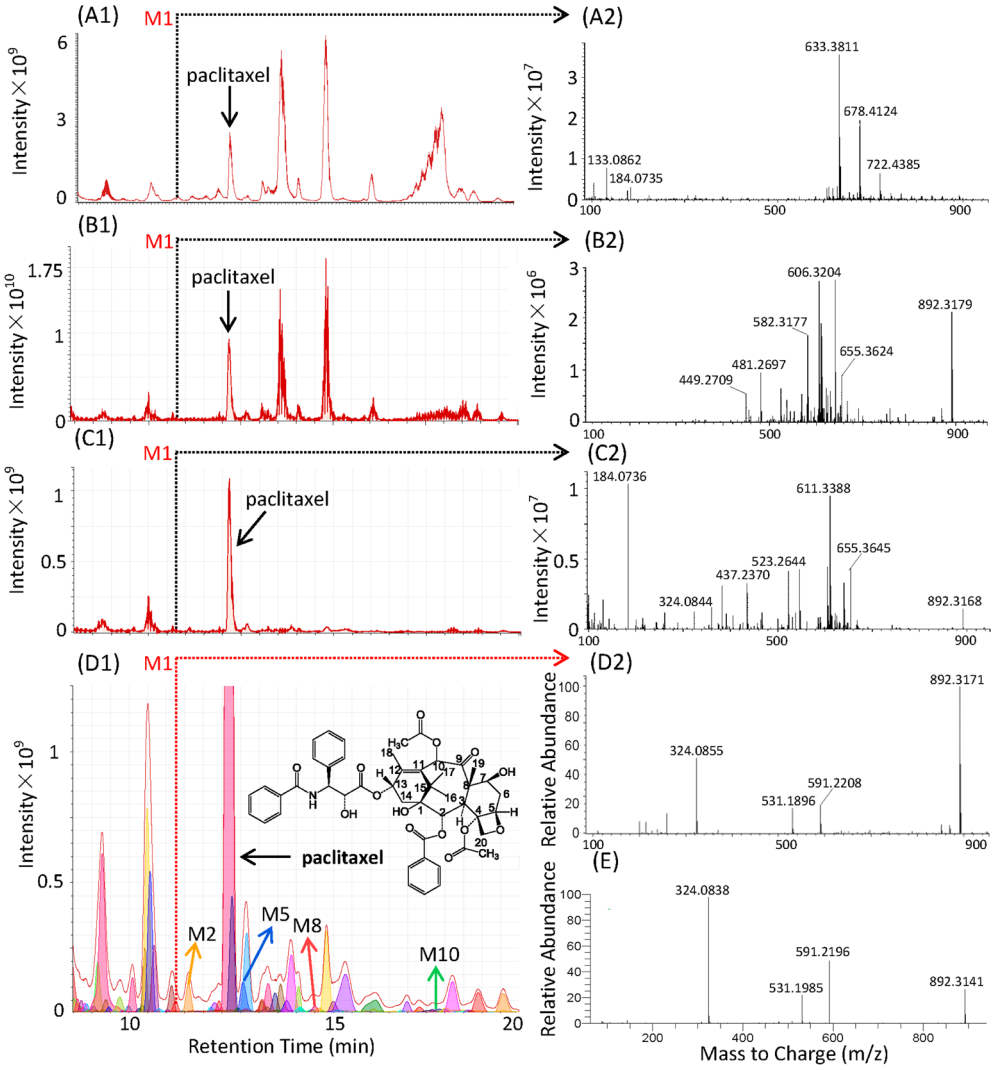

Figure 4. Ion chromatograms and tm-MS spectra (at 10.81 min) acquired by a tm-MS scan with different data-processing methods. (A1, A2) raw data; (B1, B2) processed by MDF (parameters: template mass, m/z 876.3202; ΔmDa range, ± 50 mDa; filter mass range, m/z 100−1300); (C1, C2) processed by MF-MDFs; (D1, D2) processed by MF-MDFs coupled with blind deconvolution; (E) HRMS/MS spectrum of M1 (at 10.81 min) acquired by a traditional MS/MS scan.

plasma, of which six were not previously reported. We identified 10 paclitaxel metabolites in female BALB/c-nu mice that formed by phase I metabolic processes (Table S2, Supporting Information). The chemical structures of the 10 metabolites were confirmed based on the HRMS/MS spectrum, according to structural features of taxoids and metabolites identified in a comprehensive study of paclitaxel metabolism (Scheme S1, Supporting Information). Four metabolites (M1, M2, M3, and M8), which were detected in mouse, rat, and human, were also found in our study. For M1, the assigned positions of hydroxyl groups were confirmed by 3′-hydroxypaclitaxel analytical standards. For monohydroxylated metabolites, the characteristic ions, $[ScH + Na]^+$ at m/z 308.0899, $[M + Na - ScH + O]^+$ at m/z 607.2155, and m/z 409.1617, indicated that M3 was 2m-hydroxypaclitaxel, whereas the characteristic ions at m/z 485.1776 and m/z 425.1566 indicated that hydroxylation occurred at C6, C16, C17, or C19. Because neither M5 nor M8 had the same retention time as the 6α-hydroxypaclitaxel analytical standards, these two metabolites were speculated to be 16 (or 17)-hydroxypaclitaxel and 19-hydroxypaclitaxel. Further structural elucidation is provided in Figure S3, Supporting Information.

CONCLUSIONS

In this study, we proposed a novel TDIAM strategy for the in-depth discovery and identification of in vivo metabolites using UHPLC-HRMS/MS. The results demonstrated that the TDIA strategy is a sample- and time-saving method to collect comprehensive and targeted information for metabolite research in only one injection, and that it provides structure-enriched evidence (including molecular ion and fragment ions) to confirm the existence of and elucidate the structure of metabolites. More importantly, as the S/N values of parent ions and product ions of potential metabolites were significantly increased with TDIA, this method effectively reduced interference from endogenous components and greatly improved detective sensitivity and selectivity. On this basis, TDIM, an innovative data-processing method using MF-MDFs and blind deconvolution, was established to take full advantage of TDIA. TDIM could effectively pick out molecular ions and fragment ions from complex data to yield almost the same

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spectrum as traditional HRMS/MS. When we applied our proposed strategy to a study of paclitaxel metabolism, we successfully identified 10 metabolites of paclitaxel, among which six were not previously reported. Thus, this strategy represents one that is more comprehensive, sensitive, and specific for identification of low-level metabolites in a complex matrix. This strategy can be used as a powerful tool for research in drug metabolism, targeted metabolomics, and proteomics.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text, including methods and supplementary tables and figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01205.

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Notes

The authors declare no competing financial interest.

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