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Short communication

Structural identification of the metabolites for strictosamide in rats bile by an ion trap-TOF mass spectrometer and mass defect filter technique

Yan Liang[a], Wei Xiao[b], Chen Dai[a], Lin Xie[a], Gang Ding[b,c,*], Guangji Wang[a,**], Zhaoqing Meng[b,c], Juan Zhang[b,c], An Kang[a], Tong Xie[a], Yanna Liu[a], Yuanyuan Zhou[a], Wenjun Liu[b,c], Li Zhao[b,c], Jia Xu[b,c]

[a] Key Lab of Drug Metabolism & Pharmacokinetics, China Pharmaceutical University, No. 24, Tongjiaxiang Street, Nanjing 210009, China
[b] Jiangsu Kanion Pharmaceutical Co. Ltd., 58 Haichang South Road, Xinpu District, Lianyungang 222001, China
[c] Jiangsu Zeukov Pharmaceutical S.&T. Inc., 1706 Shuanglong Road, Jiangning District, Nanjing 211100, China

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ABSTRACT

We report herein, a facile metabolite identification workflow on the antimicrobial strictosamide, which is derived from accurate mass measurement by a hybrid ion trap-TOF mass spectrometer. In step 1, the parent drug and metabolites in rat bile were separated on an HPLC column followed by ion trap-TOF mass spectrometer analysis after a single oral dose of 50 mg/kg strictosamide. In step 2, mass defect filter technique, which enables high-resolution mass spectrometers to be utilized for detecting drug metabolites based on well-defined mass defect ranges, was used to find metabolites in the mass spectrum. In step 3, the differences of accurate masses and their mass fragmentation pattern among the parent drug and metabolites used to assign structures for the metabolites successfully. As a result, five metabolites of strictosamide were found in rat bile, and all the metabolites were reported for the first time.

1. Introduction

Drug metabolite identification by LC/MS techniques always involves two steps: (1) detection of drug metabolite ions in a bio-fluid, (2) acquisition of their MS^1 and MS^n spectra for structural characterization [1]. In general, drug metabolites can be categorized into common (predictable) and uncommon (unpredictable) metabolites. Common metabolites are those formed by conventional biotransformation reactions such as O-methylation, hydroxylation and N-dealkylation, whose molecular masses can be predicted easily [2,3]. Uncommon metabolites are those formed via unpredictable biotransformation reactions, and the mass shifts are difficult to predict. Recently, a mass defect filter (MDF) technique was developed for detecting drug metabolites via post-acquisition processing of high-resolution LC/MS data. The key to the creation of MDF algorithm was the realization that the mass shifts of metabolite ions typically fall within ±50 mDa relative to that of the parent drug. When such a filter (e.g. ±50 mDa from the mass defect of the parent drug) was applied to high-resolution LC/MS data, the majority of interference ions were automatically removed, and the resulting simplified data could facilitate the identification of drug metabolite ions [3,4].

Strictosamide was the (3α, 15β, 16β, 17α)-21-oxo-16-vinil-19,20-dihydro-oxoyoimban-17-il-β-D-glucopyranoside, a glycoalkaloid found in *Sarcocephalus latifolius* (Smith) Bruce (*Nauclea latifolia* Sm.) which exhibit remarkable antimicrobial and antiparasitical activities [5,6]. Experiments with *Sarcocephalus latifolius* showed that strictosamide is the most abundant alkaloid isolated from its roots and may be responsible for the slight hypotensive action and the negative inotropic and chronotropic reversible effects produced on frog and rabbit isolated heart [7,8]. In 2010, Hu et al. described a method using LC–MS/MS to investigate its pharmacokinetic characterize in rat [9]. In this study the use of LC-IT-TOF-MS-generated MS and MS^n data were utilized to rationalize metabolites of strictosamide.

2. Experimental

2.1. Chemicals and reagents

Strictosamide (purity > 95%) was supplied by the Kanion Pharmaceutical, and ranolazine (purity >99%; internal standard) was purchased the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile and methanol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethyl acetate and formic acid were analytical grade. Ultrapure water was prepared by the Milli-Q Ultrapure water purification sys-

* Corresponding author at: Jiangsu Kanion Pharmaceutical Co. Ltd., 58 Haichang South Road, Xinpu District, Lianyungang 222001, China. Tel.: +86 25 86587928; fax: +86 25 86587926.
** Corresponding author. Tel.: +86 25 83271128; fax: +86 25 85306750.
E-mail addresses: dingg2000@126.com (G. Ding), guangjiwang@hotmail.com (G. Wang).

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tem (Millipore, Bedford, MA, USA). Other chemicals and solvents were all of analytical grade.

2.2. Sample collection and preparation

2.2.1. Experimental animals

Animal studies were conducted according to protocols approved by the Review Committee of Animal Care and Use. Six male Sprague-Dawley rats (weighing 200–250 g) were fasted for 16–22 h with free access to water. A cannula was implanted under ether anesthesia for the entire duration of the surgery for the bile collection. The blank bile was collected before administration of strictosamide. After recovery from the surgery, the pre-prepared drug solution in water was oral administrated to the rats at a dose of 50 mg/kg. The bile was collected in Eppendorf 15 mL microcentrifuge polypropylene tube for 12 h, and the rats were given water occasionally during the course of bile collection. All these samples were stored at −20 °C until analysis.

2.2.2. Purification of bile samples

Blank and administrated bile samples (200 μL) were diluted with 1.0 mL methanol, and the methanol effluent was centrifugated at 40,000 × *g* for 10 min at 4 °C, the supernatants were stored at −20 °C until for LC-IT-TOF/MS analysis.

2.3. Instrumentation and conditions

HPLC experiments were conducted on a Shimadzu (Kyoto, Japan) HPLC system consisting of an LC-20AB binary pump, a DGU-14A degasser, a SIL-20AC autosampler and a CTO-20AC column oven. Chromatographic separation was achieved on a Thermo ODS 5 μm 50 mm × 2.1 mm ID column at 35 °C (Thermo, USA). The mobile phase (delivered at 0.2 mL/min) comprised solvent (A), ultrapure water containing 0.02% HCOOH and solvent (B), acetonitrile. A gradient elution was performed: initial 10% B for 0.5 min, linear gradient 10–80% B from 0.5 to 15 min and retained until 17 min then quickly returned to initial 10% B in 1 min and maintained for a further 4 min for column balance.

MS^n analyses were conducted on a Shimadzu IT-TOF-MS (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source, and the optimized operating conditions were as follows: positive mode; electrospray voltage, 4.5 kV; nebulizer gas (N_2) flow, 1.5 L/min; nebulizer gas (N_2) flow, 5 L/min; trap cooling gas (Ar) flow, 95 mL/min; pressure of ion trap, 1.7×10^{-2} Pa; pressure of TOF region, 1.5×10^{-4} Pa; Ion accumulated time, 30 ms; collision energy was set at 50% both for MS^2 and MS^3; scan range of *m/z* 250–700 for MS^1, 100–600 for MS^2, 50–500 for MS^3.

Metabolites identification was performed by MetID solution 1.0, and Shimadzu's Composition Formula Predictor software was used to provide chemical formula for strictosamide and its metabolites.

3. Results and discussion

The full scan mass spectra of rat bile after administration of strictosamide were compared with those of blank samples to find the possible metabolites with the aid of MetID solution software. Fig. 1 shows the EICs of a blank bile sample and a dosed bile sample of rat. Besides the parent drug, there were five new peaks which could be attributed to metabolites derived from the oxidation, reduction, de-glycosylation of strictosamide. Possible metabolite structures were considered based on the structure of strictosamide and known common metabolic pathways.

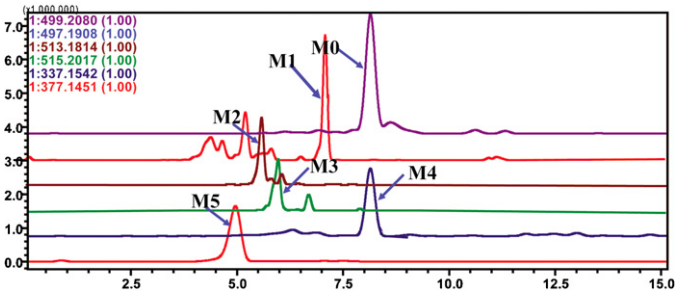

Fig. 1. Extracted ion chromatograms (EIC) of a dosed bile sample of rats.

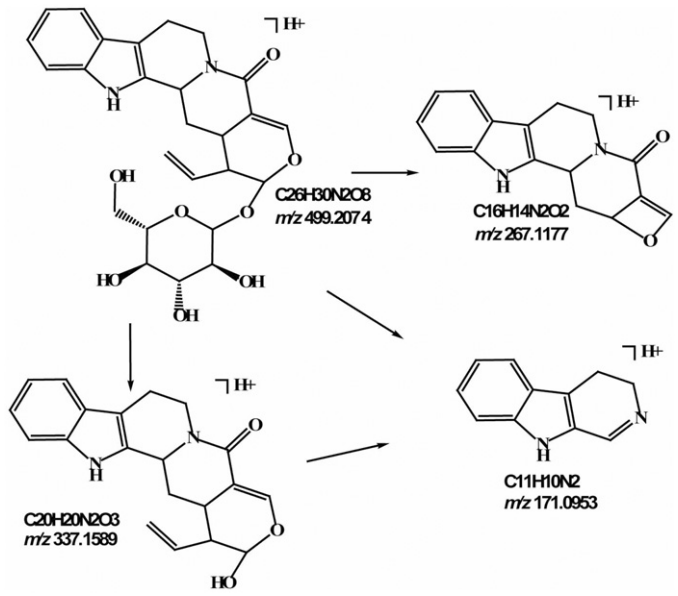

Fig. 2. The proposed fragmentation pathways of strictosamide.

3.1. Fragmentations of strictosamide

To ensure sufficient fragments production, 500 ng/mL of strictosamide prepared in methanol was used for the fragmentation pattern study. Accurate mass measurements of protonated molecule and fragment ions of strictosamide are presented in Table 1. The $[M+H]^+$ ion at *m/z* 499.2074 ($C_{26}H_{30}N_2O_8$) was observed as the predominant protonated molecule ion. The product ion at *m/z* 337.1589 ($C_{20}H_{20}N_2O_3$), the loss of a glucose (Glu), is one of the major fragments. The fragment at *m/z* 267.1177 ($C_{16}H_{14}N_2O_2$) was formed from the loss $C_{10}H_{16}O_6$ from the parent drug. Besides, the parent drug and the fragment at *m/z* 337.1589 can both produce 4, 9-dihydro-3H-beta-carboline (*m/z* 171.0953, $C_{11}H_{10}N_2$) through further fragmentation. The proposed fragmentation pattern of strictosamide is illustrated in Fig. 2.

3.2. Structural determination of metabolites

The protonated molecule ion at *m/z* 497.1908 (M1, $C_{26}H_{28}N_2O_8$) and its MS^2 ion at *m/z* 335.1430 were both 2 Da less than those of strictosamide, respectively. These results indicated that M1 was the dehydrogenation product of strictosamide. On the basis of the elemental compositions of product ions and bond connectivities present in the parent molecule, the most likely dehydrogenating position was located at the tetrahydro-pyridine ring. Importantly, its MS^3 ion at *m/z* 169.0842 was also 2 Da less than that (4,9-dihydro-3H-beta-carboline, *m/z* 171.0953) of strictosamide, and this product ion further confirmed the reliability of the inference

Table 1
Retention time, formula, accurate mass and mass error of strictosamide and its metabolites in rat bile detected by LC-IT-TOF-MS.

No.	Parent ion *m/z* (ppm error)	Retention time (min)	Formula	Product ions (MS^n)
M0	499.2080 (1.2)	8.1	$C_{26}H_{30}N_2O_8$	337.1589; 267.1177; 171.0953
M1	497.1908 (3.40)	7.1	$C_{26}H_{28}N_2O_8$	335.1430; 263.0864, 169.0842
M2	513.1814 (1.17)	5.5	$C_{26}H_{28}N_2O_9$	281.1001; 263.0942; 261.0620; 247.0837; 235.1048; 206.0706
M3	515.2017 (0.14)	6.0	$C_{26}H_{30}N_2O_9$	335.1464; 282.0907
M4	337.1542 (0.89)	8.3	$C_{20}H_{20}N_2O_3$	267.0963; 171.0885
M5	377.1451 (1.33)	5.0	$C_{22}H_{20}N_2O_4$	243.0876; 198.0618; 172.0882

above. The possible structure and proposed fragmentation pathways of M1 are shown in Fig. 3(a).

M2, another main metabolite appeared in bile, was calculated as $C_{26}H_{28}N_2O_9$ by the Formula Predictor software according to the accurate mass. Thus, M2 was preliminary concluded as the hydrolation product of M1. The spectra of MS^2 obtained from the precursor ion at *m/z* 513.1814 showed five major product ions at *m/z* 281.1001 (base peak), 263.0942, 247.0837, 235.1048 and 206.0706. From the product ions at *m/z* 281.1001, the hydroxylation was assigned on the nucleus. According to the product ions at *m/z* 263.0942 and 281.1001, hydroxylation should not occurred on the ring bond because of the loss of one H_2O. In the MS^3 experiment, isolation and fragmentation of the product ion at *m/z* 281.1001 resulted in one ion at *m/z* 206.0706 from a loss of benzene. Thus, the hydroxylated position was located on 2-carbonyl-tetrahydropyridine ring. The structure and the fragment pathway have been concluded and are shown in Fig. 3(b).

Likewise, metabolite M3 was calculated as $C_{26}H_{30}N_2O_9$ by the Formula Predictor software according to the accurate mass measurement. The spectra of MS^2 obtained from the precursor ion at *m/z* 515.2017 showed two major product ions at *m/z* 335.1461 (base peak) and 282.0907. The major MS^2 ion at *m/z* 335.1461 was 2 Da less than that of strictosamide. These results indicated that dehydrogenation, as same as M1, occurred on the nucleus of M3, and hydrolysis reaction occurred on the glucosyl group. The fragment ion (*m/z* 282.0907) confirmed the reliability of the previous inference, and the possible structure and proposed fragmentation pathways of M3 are shown in Fig. 3(c).

M4, eluted at 8.3 min, showed the predominant quasimolecular ion $[M+H]^+$ at *m/z* 337.1542 ($C_{20}H_{20}N_2O_3$) that is a $C_6H_{10}O_5$ less than the parent compound strictosamide, suggesting that it was a de-glucose product of strictosamide. The fragment ion at *m/z* 267.0963 and 171.0885 were in excellent agreement with the parent compound. Therefore, the M4 was been

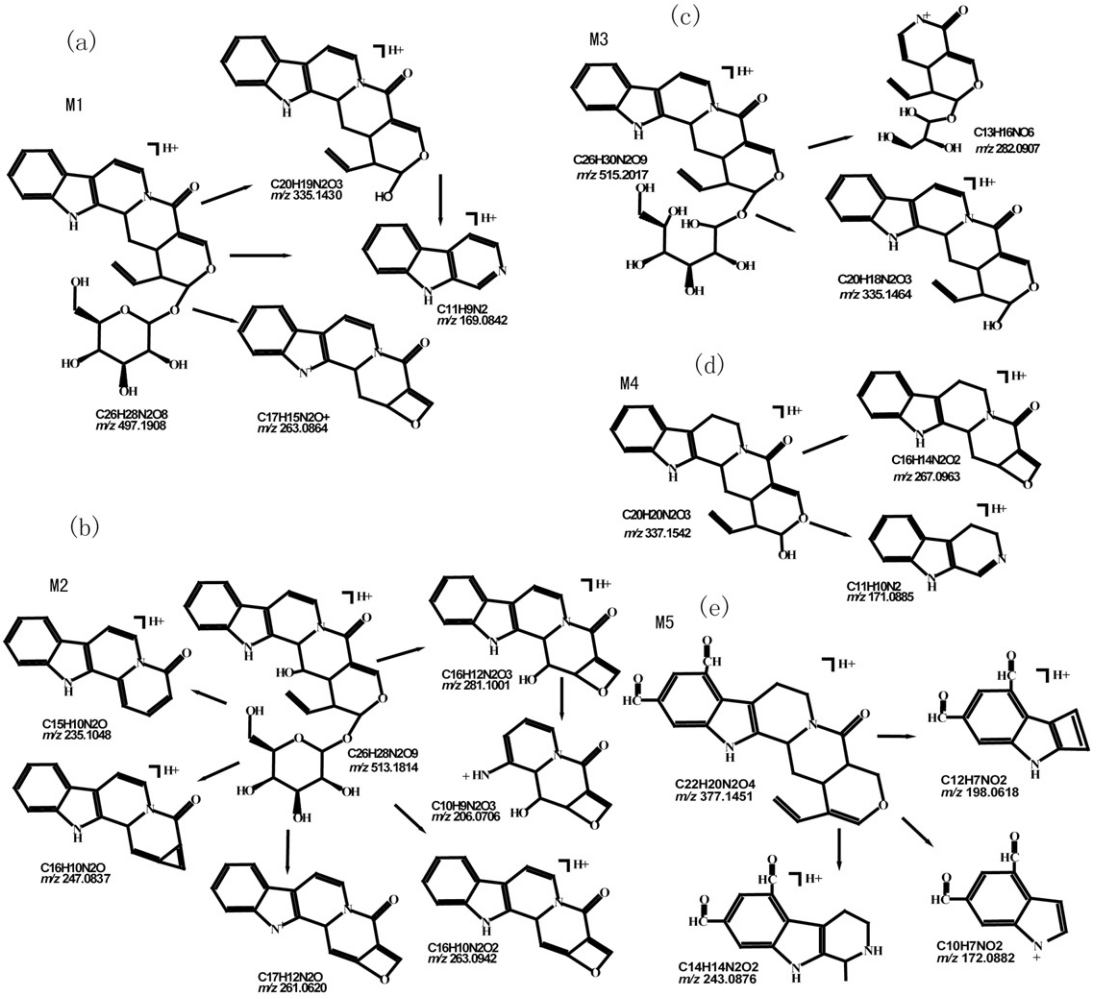

Fig. 3. Proposed fragmentation mechanism of M1 (a), M2 (b), M3 (c), M4 (d) and M5 (e).

rapidly characterized as the de-glucose product of strictosamide (Fig. 3(d)).

M5, eluted at 5.0 min, showed the predominant protonated molecule ion $[M+H]^+$ at m/z 377.1451, and was calculated as $C_{22}H_{20}N_2O_4$ by the Formula Predictor software. The further fragmentation patterns and retention behavior analysis helped us to characterize the structure of M5. According to the elemental composition, de-glucose should occurred in the metabolic process. When matching the formula of M5 with nucleus of the parent compound, a 56 Da difference corresponding to C_2O_2 had been added on the nucleus. Therefore, M5 was tentatively proposed to be a bi-formaldehyde and de-glucose product of strictosamide. The major fragment ion at m/z 172.0882 suggested that the bi-formaldehyde was occurred on the benzene moiety. The fragmentation pattern of M5 supported such a characterization (see Fig. 3(e)).

4. Conclusion

The metabolites of strictosamide in rat urine have been studied for the first time by LC-IT-TOF-MS. The MS^n data with high mass accuracy provided much formation to investigate the structures of metabolites under electrospray ionization. The major metabolic pathways of strictosamide in rat urine were found to be dehydrogenation, hydrolation and dehydrogenation, hydrolation, de-glucose and de-glucose and bi-formaldehyde. Furthermore, the experimental results indicate that the IT-TOF mass spectrometer is powerful and effective tool for in the detection and characterization of drug metabolites in samples of biological origin.

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