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Rapid identification of bile acids in snake bile using ultrahigh-performance liquid chromatography with electrospray ionization quadrupole time-of-flight tandem mass spectrometry

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ABSTRACT

Snake bile, a precious traditional Chinese medicine (TCM), was used as the major ingredient of some Chinese patent drugs, such as Shedan Chuanbei powder and Shedan Chenpi powder for hundred years. However, there is still requirement for the comprehensive and definite composition of bile acids in snake bile. In order to rapidly identify the bile acids constituents in snake bile to avoid the adulteration, ultrahigh-performance liquid chromatography with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UPLC/ESI-QTOF-MS/MS) has been applied to conduct a qualitative analysis on snake bile acids. ESI ion source was used for mass spectra, and data were collected in both positive and negative ion mode. 16 kinds of reference standards, attributed to free bile acids, taurine- and glycine- conjugated bile acids, were detected and their MS behaviors were summarized. In negative ion mode, the diagnostic ions of free bile acids were obtained via the neutral losses of H_2O and CO_2 molecules; the diagnostic ions of taurine-conjugated bile acids were at *m/z* 124.0068 ($[C_2H_6NO_3S]^-$), *m/z* 106.9803 ($[C_2H_3O_3S]^-$) and *m/z* 79.9568 ($[SO_3]^-$); the diagnostic ion of glycine-conjugated bile acids was at *m/z* 74.0242 ($[C_2H_4NO_2]^-$). In positive ion mode, dehydration ions, amide bond cleavage ions, and reversed Diels-Alder at A-ring ions were detected in every kind of reference. These reference MS behaviors were used for identifying bile acids without reference standards in snake bile. As a result, totally 15 compounds, including 4 pairs of isomers, were identified by comparing the retention time, exact molecular mass and fragmentation behaviors with reference standards, respectively. Tauro-3β,7α,12α-trihydroxy-5β-cholenoic acid, Tauro-$\triangle^8$-3β,7α,12α-trihydroxy-5β-cholenoic acid, Tauro-3α,7α,12α,23*R*-tetrahydroxy-5β-cholenoic acid, and Tauro-3α,7α-dihydroxy-12-oxo-5β-cholenoic acid, Taurocholic acid, Glycocholic acid, Taurochenodeoxycholic acid, Taurodeoxycholic acid and Cholic acid were detected as the common bile acids in snake bile. Tauro-$\triangle^8$-3β,7α,12α-trihydroxy-5β-cholenoic acid, Tauro-3α,7α,9α,16α-tetrahydroxy-5β-cholenoic acid, Tauro-3α,12α,17*R*,22*R*-tetrahydroxy-5β-cholenoic acid, and Tauro-$\triangle^{1,8}$-3β,7β,12α-trihydroxy-5β-cholenoic acid were firstly reported in this study.

1. Introduction

Snake is a kind of significant traditional Chinese medicine (TCM). *Agkistrodon* (Qishe), *Zaocys* (Wushaoshe) and *Bungarus Parvus* (Jinqianbaihuashe) are used to expel wind, unblock the meridians and relieve convulsion for the treatment of pain from wind-dampness, spasm of the muscles and tendons, tetanus and hemiplegia. Periostracum serpentis (Shetui) has the effect of eliminating nebula and improving eyesight [1]. Snake venom (Shedu), a liquid secreted from the viper gland, is effective in analgesiac [2], and antibiosis [3]. Snake oil (Sheyou), a skincare product refined from snake belly fat, is often used for empyrosis, chapped skin, and chronic eczema. Snake bile (Shedan), which has been used as a precious

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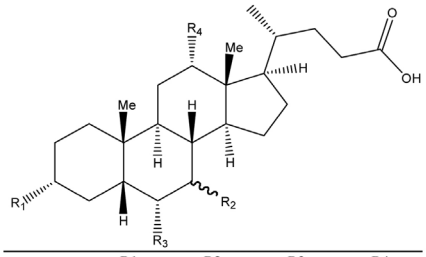
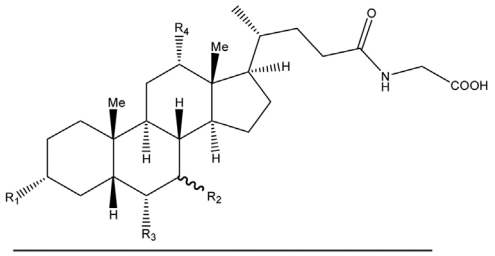
TCM for hundred years, is commonly used for the treatment of fever, cough, eclampsia and gastrointestinal diseases [4,5]. There are more than 20 preparation in Chinese Pharmacopoeia(2015) using snake bile as the main raw material, including well-known Chinese patent medicine Shedan Chuanbei powder (tablets, capsules, soft capsules), Shedan Chenpi powder (tablets, capsules), Pian Zaihuang (pastilles, capsules) and so on [6]. According to preliminary investigation, the mainstream varieties of snake bile in TCM are nearly from ten species of three families, including *Naja naja*, *Bungarus fasciatus* and *Bungarus multicinctus* of family Elapidae; *Ptyas korros*, *Ptyas mucosus*, *Elaphe carinata*, *Zaocys dhumnades*, *Enhydris chinesis* and *Dinodon rufozonatum* of family Colubridae; *Agkistrodon halys* and *Agkistrodon acutus* of family Viperidae. Due to the disordered headstream and lacking of quality standard, the quality of preparation of snake bile is not effectively controlled and it is common to see snake bile adulteration from other animal bile in market. To solve this issue, the main constituents in snake bile should be elaborated. Bile acids (BAs), a kind of steroids bearing a carboxyl group at C-17 side chain, are the main active ingredients of snake bile and play a significant role in cholesterol catabolism and lipid absorption [7,8]. According to previous reports, hundreds of bile acids have been detected, including basic BAs (Cholic acid, Deoxycholic acid, Chenodeoxycholic acid, Ursodesoxycholic acid, Hyodeoxycholic acid, Lithocholic acid, and taurine and glycine-conjugated BAs) [9], and their oxidized BAs, unsaturated BAs, as well as their derivatives combined with glucuronic acid, sulphuric acid, glucose and so on [8,10], while only five BAs, namely Taurocholic acid (TCA), Taurochenodeoxycholic acid (TCDCA), Taurodeoxycholic acid (TDCA), Tauropythocholic acid (TPCA), and Glycocholic acid (GCA) [11,12] in snake bile were diagnosed by using thin layer chromatography (TLC), capillary electrophoresis (CE) [13], capillary gas-liquid chromatography(CGC) [14] and high-performance liquid chromatography (HPLC) methods [15]. However, those methods were not sensitive and selective enough to accurately analyze the trace BAs in snake bile.

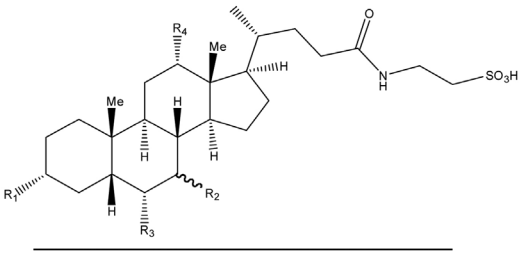
Recently, High-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI–MS/MS) has been successfully used to elucidate BA constituents in various bile, including basic BAs [16] and their derivatives such as Sulfates BAs [17], 3β-Hydroxy-Δ^5-bile acid [18], 3-keto-cholanoic acid [19], 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5β-cholenoic acid [20] and so on. Generally, triple quadrupole mass spectrometry (QQQ-MS) is often used for quantitative analysis by MRM form [18–20]. And quadrupole time-of-flight tandem mass spectrometry (Q-TOF-MS) is often used for qualitative analysis because it has a higher resolution and can provide more accurate mass measurements. The molecular formula of a compound can be confirmed by accurate mass date [21]. The application of Q-TOF-MS could obtain accurate masses of molecular ions and detailed fragmentation information to remove ambiguous interpretation and facilitate structure elucidation [22]. Therefore, for analysis of BAs without reference, Q-TOF-MS/MS is a preferred technique because of the capability to provide additional selectivity required for unambiguous identification [23–25]. Herein, UPLC/ESI-QTOF-MS/MS method, both in negative and positive ion mode, was employed to analyze BAs in snake bile [25]. In present study, sixteen reference BAs were analyzed by UPLC/ESI-QTOF-MS/MS. Their structures were shown in Fig. 1. Their retention time and key diagnostic fragment ions and possible pathways of fragmentation had been detected and summarized. Therefore, the structure of BAs in snake bile could be elucidated based on the retention time and the fragmentation behaviors of these reference compounds.



	R1	R2	R3	R4
CA	OH	α-OH	H	OH
DCA	OH	H	H	OH
CDCA	OH	α-OH	H	H
UDCA	OH	β-OH	H	H
HDCA	OH	H	OH	H
LCA	OH	H	H	H



	R1	R2	R3	R4
GCA	OH	α-OH	H	OH
GDCA	OH	H	H	OH
GCDCA	OH	α-OH	H	H
GHDCA	OH	H	OH	H
GLCA	OH	H	H	H



	R1	R2	R3	R4
TCA	OH	α-OH	H	OH
TDCA	OH	H	H	OH
TCDCA	OH	α-OH	H	H
TUDCA	OH	β-OH	H	H
THDCA	OH	H	OH	H

Fig. 1. Structures of reference standard.