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Separation and identification of norcantharidin metabolites in vivo by GC–MS method

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ABSTRACT

Norcantharidin (NCTD), the demethylated analogue of cantharidin, inhibits the proliferation of a variety of human tumor cell lines, and appears to cause the least nephrotoxic and inflammatory side effects. Although NCTD has been used to treat human cancers in China for years, there is no report regarding its metabolism up to now. This is the first report to separate and identify the main metabolites of NCTD in vivo by GC–MS using TMS derivatives. Two hydrolyzed products and five phase I or phase II metabolites were found in rat by the chromatogram comparisons of the blank with incurred biological samples. Multiple stages of fragmentation patterns were used to confirm the metabolites characterizations. The established GC–MS method can also be applied to identifying unknown metabolites of the drugs containing hydroxyl or carbonyl groups in molecular structure.

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1. Introduction

Blister beetle (*Mylabris phalerata Pall.* or *M. cichoril* L.) has been used in traditional Chinese medicine for the treatment of malignant tumors by its main content cantharidin for more than 1000 years. Norcantharidin (NCTD, (1S,2R,3S,4R)-7-oxabicyclo-[2,2,1] heptane-2,3-dicarboxylic anhydride), the demethylated analogue of cantharidin, inhibits the proliferation of a variety of human tumor cell lines [1–11], and appears to cause the least nephrotoxic and inflammatory side effects. Moreover, NCTD is not a substrate for the P-glycoprotein pump, a known mechanism for developing cancer resistance [6,7]. It is noteworthy that NCTD can stimulate the bone marrow and increase the peripheral white blood cell significantly [12], which makes it more valuable in combination chemotherapy since most of the chemotherapeutic agents in use today have the side effect of bone marrow suppression.

Although NCTD was used to treat human cancers in China for years [13], there was no report describing the metabolic profiles of NCTD. Early studies hinted that the promoted biotransformation occurred in vivo after oral administration of NCTD [14].

Despite the recent advancement of various analytical tools, the metabolite identification for compounds undergoing multiple and unpredictable metabolism from biological matrix still remains great challenges [15]. In the past few years, mass spectrometry coupled with chromatographic separation has become a powerful and frequently used technique for metabolite separation and identification. GC–MS remains a technique of choice for the metabolic profiling of biological matrix due to a better discrimination of the compounds in the gas than in the liquid phase, capability for unknown compound identification based on the fragmentation pattern and well-established databases, which can offer the structure information of unknown with standard library retrieval to be applied to the identification and recognition of new compounds. In addition, the higher sensitivity of GC–MS compared to LC–MS could allow for the quantification of metabolites that are in small quantities. These, and other advantages, render GC–MS a useful tool for metabolites molecular diagnosis.

According to the chemical structure of NCTD (Fig. 1), the anhydride group and the oxo-bridge were easily hydrolyzed to carboxyl and hydroxyl metabolites, which can react with silylation reagents to form more volatile and thermo-stable silane derivatives. In this study, the rat serum and bile samples after dosing of NCTD were

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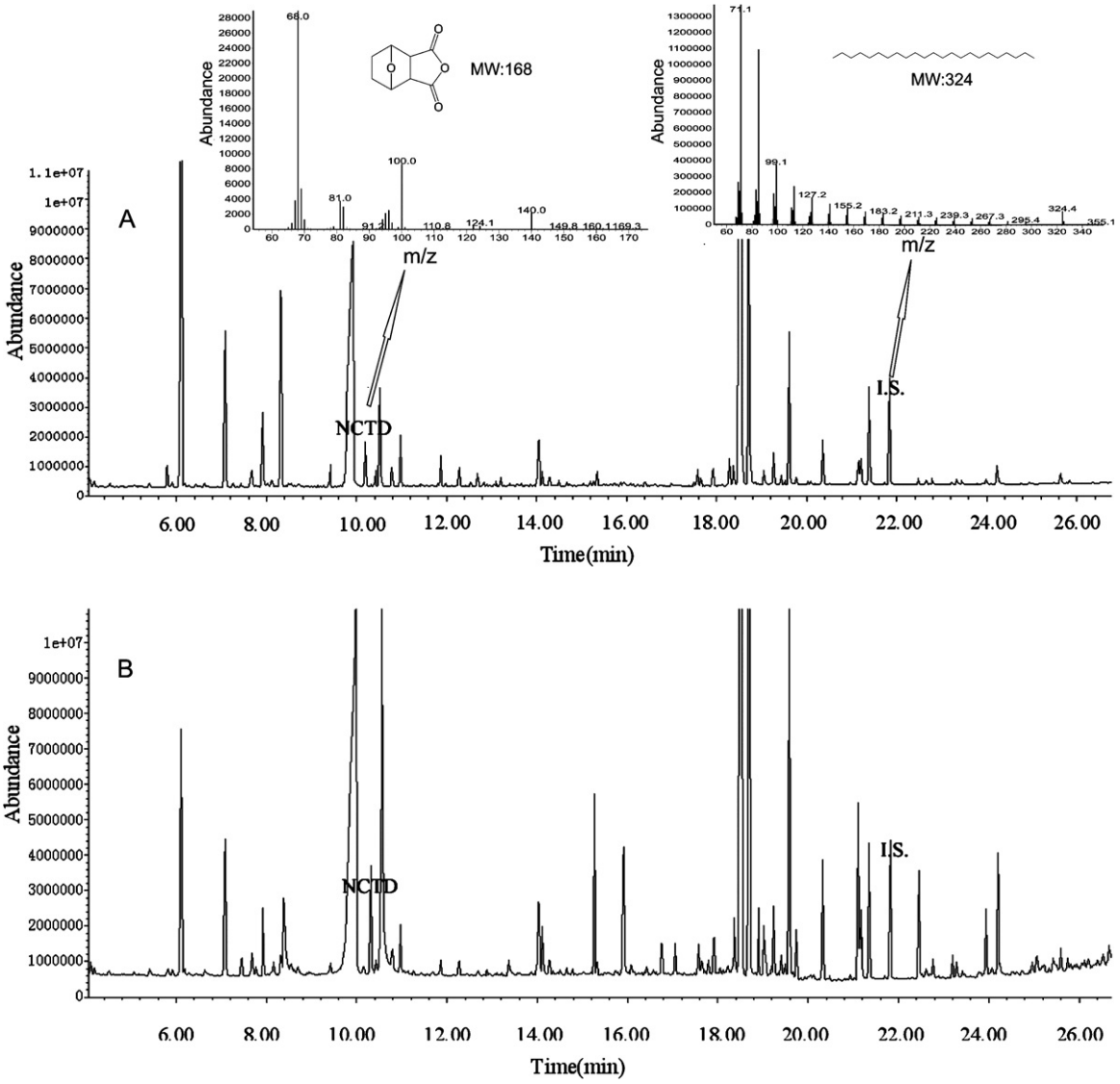

Fig. 1. The total ion chromatograms of rat serum (A) and bile (B) samples spiked with NCTD and I.S. (n-tricosane), and the mass spectrum of NCTD and I.S.

separated and identified by GC–MS technology to investigate the characteristics of NCTD metabolites in vivo. It revealed that seven main metabolites existed in rat, and some of them were newly detected. The results could be useful for future studies involving NCTD, such as clinical therapy and new formulation development.

2. Materials and methods

2.1. Chemical reagents

NCTD (99.92%) was obtained from *Junan Pharmaceutical Factory* (Shandong, China). Trimethyl-chlorosilane (TMCS) was from *Sinopharm Chemical Reagent Ltd.* (Shanghai, China). O-methylhydroxylamine hydrochloride (99%) was from *J&K Chemical Ltd*. and n-tricosane (99%) was from *Johnson Matthey Company* (USA). N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, 97%) and n-heptane (99%) were from *Acros Organic* (NJ, USA). Pentobarbital sodium was purchased from *Shanghai Kefeng Chemical Reagents Co. Ltd*. (China), and pyridine was from *Tianjin Kermel Chemical Reagent Co. Ltd.* (China). HPLC grade chloroform, ethyl acetate and acetonitrile were purchased from *J.T. Baker* (USA).

2.2. Animals

Sprague-Dawley rats (200–250 g) were obtained from *the Experimental Animal Center of Shandong University* and housed with unlimited access to food and water except for fasting 12 h before experiment. All procedures were pre-approved by the Institutional Animal Use Committee.

2.3. Drug administration and sample collection

Eighteen rats were divided into three groups. 100, 150 mg kg^{-1} of NCTD solution (10 mg mL^{-1}) and 3 mL saline were intragastrically given at fasting conditions. All rats were anaesthetized by subcutaneous injection of 3% pentobarbital sodium solution (1 mL/100 g). 200–400 μL bile samples were drained at pre-dose and 0–2 h, 2–4 h, 4–6 h post-dose. 800 μL of blood were taken from sinus jugularis before and 15 min, 30 min, 45 min, 1 h and 1.5 h

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