LC-MS ANALYSIS OF TRICLOSAN AND ITS METABOLITES: DIOXINS RELATED?

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Abstract

Triclosan is a widely used broad-spectrum antibacterial agent by specifically inhibiting enoyl–acyl carrier protein reductase. The oral metabolism of triclosan was investigated on SD rat, while *in vitro* metabolic study was performed using SD rat liver S9 and microsome. Fourteen metabolites were identified by using in-source fragmentation from high performance liquid chromatography-negative atmospheric pressure chemical ionization/ion trap mass spectrometry (HPLC-APCI/ITMS). The results of *in vivo* metabolism indicated that the glucuronidation and sulfonation were the major pathway of phase II metabolism and the hydroxylated products were the major phase I metabolites. Moreover, glucose, mercapturic acid and cysteine conjugates of triclosan were also observed in the urine samples of rats orally administrated with triclosan. The *in vitro* metabolic rates of triclosan and its major glucuronidation and sulfonation metabolites were determined by isotope dilution high performance liquid chromatography-electrospray/ion trap mass spectrometry (HPLC-ESI/ITMS) method.

Introduction

Triclosan is an antimicrobial agent against both Gram-positive and Gram-negative bacteria with its antifungal and antiviral properties. The chemical has been extensively used in pharmaceutical and personal care products, including antiseptic soaps, toothpastes, mouthwashes, cosmetics, dermatological agents, fabrics, and plastics. Triclosan and its derivatives were often detected in environmental water and sediments [1-2] as well as biological fluids including milk, plasma and urine [3]. The research results on the toxicity of triclosan were not consistent. While triclosan has low acute toxicity for mice with LD_{50} values of >1 g/kg [4], the chemical was also reported to be a selective inhibitor of the glucuronidation and sulfonation of phenolic xenobiotics and a potent inhibitor of pentoxyresorufin *O*-dependentylasethoxyresorufin O-deethylase [5].

There have been a few reports on triclosan metabolism and only sulfate and glucuronide conjugates were detected in mammals. Triclosan was excreted primarily as the glucuronide conjugate following the application of a 1% formulation to guinea pig or rat skin [6]. Sulfate and glucuronide conjugates of triclosan were formed in human liver enzymes *in vitro* as well as human and rat skin *in vivo*, while no oxidative metabolites were detected in the urine after the exposure to triclosan [7]. The sulfate conjugate was also detected in polar bear liver enzymes *in vitro* [8]. The determination of triclosan metabolites *in vivo* and/or *in vitro* was conducted by using liquid scintillation spectrometer, fluorometric or radiochemical thin-layer chromatographic analysis and HPLC-UV [6-8]. LC-MS has been applied for the determination of triclosan in biological and environmental samples [9-10]. In this study, the

in-source fragmentation method was developed and applied for for the analysis of triclosan and its metabolites *in vivo* and *in vitro*.

Experimental

Chemicals

Triclosan and $^{13}C_{12}$-labeled triclosan were purchased from Wellington Laboratories (Ontario, Canada). HPLC grade acetonitrile and methanol were purchased from Tedia (USA). Water was collected from a Milli-Q Ultrapure water system with the water outlet operating at 18.2MV (Millipore, Billerica, USA). Uridine diphosphoglucuronic acid (UDPGA) was obtained from Wako (Japan). Rat liver S9 and microsome were prepared from male Sprague-Dawley (S.D.)

In vitro study

Glucuronidation conjugation was investigated by incubating with each 200 μl of rat liver S9 and microsome (1g rat liver /2ml of buffer solution) at 37°C for 5 min in a cofactor (795 μl) containing 80 mM Tris buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM UDPGA, as well as triplicate of 5 μl 5 mM triclosan as samples or 5 μl ethanol as control. The mixture was shaken for equilibration in a water bath at 37 °C. The reaction was stopped after the incubation for 5, 15, 45, 120 and 240 min by the addition of 1 N HCl. Two hundred μl aliquots of incubated solution were collected and extracted with 3 times of cold ethyl acetate. The samples were centrifuged by a cyclone centrifuge with freezer (Eppendorf, Hamburg, Germany) and the supernatant was collected, recombined, and evaporated to dryness by the TurboVap LV Evaporator (Zymark, Hopkinton, U.S.A.) under a stream of dry nitrogen at 40 °C. The dried residue was redissolved in 100 μl of 500 ng/ml $^{13}C_{12}$-labeled triclosan solution in acetonitrile prior to the LC-MS analysis.

LC-MS analysis

HP 1100 liquid chromatograph (Hewlett-Packard, Wilmington, DE, USA) equipped with a Waters X-terra MS-C8 reversed-phase column (100×2.1mm, 3.5 μm) was used for the LC separation. The mobile phases consisted of phase A (water, 0.5% acetic acid and 5mM ammonium formate) and B (acetonitrile, 0.5% acetic acid and 5 mM ammonium formate). The gradient program started with 30% B and held for 5min, then changed to 90% B within 15min, and held for 2min. The flow rate was 0.2 ml/min and 10 μl of sample was injected. The effluent in the first 3 min was diverted to waste to minimize the contamination of the ion source. ESI-MS were performed on a Bruker Esquire 4000 ion trap mass spectrometer (Bruker-Fransen, Bremen, Germany). Negative ion mode was used for the determination of the triclosan and its metabolites.

Results and discussion

In-source fragmentation in LC-MS analysis has been reported to provide abundant fragmentation for compounds that had poor MS/MS fragmentation. However, in-source fragmentation of triclosan was only observed for the APCI-MS experiment, probably because the source temperature of APCI was higher than ESI and the additional corona voltage applied in APCI. Although a few references indicated that higher cone voltages induced in-source fragmentation in analyzer of quadruple or triple quadruple, the corona in APCI might enhance in-source fragmentation during the ion-trap mass spectrometry analysis. The qualitative analysis of triclosan and its metabolites in the individual sample collected

from each time point and the pooled sample extracts was conducted by using both ESI-MS and APCI-MS. The SPE-treated biological sample was initially analyzed by LC-ESI-MS. Fourteen potential metabolites were detected according to the determination of corresponding deprotonated molecular or $[M–H]^-$ ions in extracted ion chromatography (EIC). The $[M–H]^-$ ion was selected as parent ion for ESI-MS/MS analysis. The in-source fragmentation, on the other hand, provided abundant fragmentations for M2 when APCI-MS analysis was conducted under the same LC conditions. In addition to the major fragment ion peak at m/z 287 corresponding to the $[M–SO_3–H]^-$ion, major fragment ions at m/z 254, 252 and 253, 251 as well as small ion peak at m/z 217 were deduced to be the products of losing chlorine of $[M–SO_3]^-$, $[M–SO_3–Cl–H]^-$, $[M–SO_3–HCl–H]^-$ and $[M–SO_3–2Cl–H]^-$, respectively. The interpretations were further confirmed from the investigation on isotope peaks. The observed isotope ratios of peaks at m/z 367, 369 and 371 as well as well the m/z 287, 289 and 291 agreed with the theoretical isotope pattern of three chlorines, while those of m/z 251 and 253 matched the fragment ion with 2 chlorines.

Phase II metabolism is usually known as conjugation of phase I metabolite with endogenous substance to form the conjugate, which was more soluble, recognizable and overall more easily excreted. Glucuronidation, sulfation, methylation, glutathione conjugation, acetylation and glucosidation were typical phase II metabolic reactions, and observed in the metabolism of triclosan in this paper.

Quantitative analysis of triclosan in *in vitro* study was performed by isotope dilution ESI-MS. In brief, in full scan mode, the ion peak of triclosan at retention time of 18.7 min was chosen as the objective. The standard curve was constructed by plotting the peak area ratios of peaks at m/z 287 for triclosan and m/z 299 for $^{13}C_{12}$-labeled triclosan versus the concentrations of standard of triclosan. The method was validated to have good linearity, recovery, accuracy and precision. About 87% and 92% of triclosan was metabolized within 240 min in S9 and microsome, respectively.

Acknowledgement

This work is supported by the Hong Kong Baptist University Faculty Research Grant (FRG) and NSFC20621703.

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