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In Vitro Metabolism Study of Resveratrol and Identification and Determination of Its Metabolite Piceatannol by LC/EC and LC/MSMS

This preliminary study focuses on tools to study the metabolism of resveratrol and potential drug-to-drug interactions from consuming wine or grape juice, which both contain resveratrol.

Introduction

Resveratrol is a naturally occurring compound in red wine and grape juice. Recent findings suggest that resveratrol may also be anticarcinogenic (1) and a potent chemopreventive agent for breast cancer (2). Previously we reported on a method for the determination of resveratrol in natural sources and for its pharmacokinetic study in rat (3,4). To continue our research on this natural product, this preliminary study is focused on tools to study the metabolism of this compound and potential drug-drug interactions from consuming wine or grape juice.

In vitro metabolism of resveratrol in rat liver microsomal incubations was investigated. The main metabolite was confirmed to be piceatannol (a known antileukemic agent) by LC and tandem mass spectrometry. This microsomal incubation result was consistent with a recent finding that resveratrol *in vitro* metabolism by recombinant human cytochrome P450 isozyme CYP 1B1 formed piceatannol (5). The metabolite piceatannol has potential anticancer activity by inhibiting an enzyme found in human tumor cells (6). ***F1*** shows the structures of resveratrol and piceatannol. Both metabolite and substrate were detected by electrospray ionization tandem mass spectrometry in the negative ion mode using full scan MS and full scan MSMS experiments. This study is the first known report that used LC/MSMS for the direct identification of a resveratrol metabolite.

Experimental

Incubation of resveratrol with rat liver microsomes was carried out at 37°C in a bench-top Lab-Line shaker (Barnstead/Thermolyne, Dubuque, IA). The incubation solution contained 50 mM potassium phosphate buffer (pH 7.4), 1 mg protein/mL microsomes, 10 mM $MgCl_2$, 2 mM NADPH and 1-50 µM resveratrol in a final volume of 200 µL. The enzyme reaction was initiated by adding NADPH after an initial 10-min preincubation. The reaction was terminated by adding 1 mL of ethyl acetate. The solution was vortex-mixed and centrifuged (Allegra™ 6R centrifuge, Beckman Coulter, Palo Alto, CA) at 4°C for 10 min at 3750 rpm. The supernatant was transferred to a test tube and dried under a stream of nitrogen at room temperature. The residue was reconstituted in 200 µL of mobile phase. The LC/MSMS system was equipped with a BASi PM-80 pump coupled to a Finnigan LCQ Deca ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA). The mass spectrometer was operated in ESI negative ion mode. A multi-channel amperometric detector (epsilon™, BASi, West Lafayette, IN, USA) was coupled to a quad-glassy carbon working electrode. Applied potentials were +400, +500, +600 and +700 mV vs. Ag/AgCl. The column was a Supecol Discovery C8, 5 µm, 150 x 2.1 mm. The mobile phase consisted of 0.5% acetic acid-acetonitrile (85:15, v/v) with a flow rate of 0.4 mL/min. A volume of 20 µL of standard solution or incubation sample was injected into LC/MS and LC/EC system.

Results and Discussion

Mass spectra in negative ion mode and a representative LC/MS chromatogram for the incubation solution are shown in ***F2***. Besides the resveratrol peak at m/z 227, the metabolite peak at m/z of 243 corresponded to its oxidation product piceatannol $(M-H)^-$. For further confirmation of the identity of piceatannol, full scan MSMS experiments were performed. ***F3*** and ***F4*** show the chromatograms and full scan MSMS mass spectra of a standards mixture and of a microsomal incubation of resveratrol, respectively. A match between the MSMS spectra of both standards and incubation solution confirms that the peak eluting at the retention time of 5 min is piceatannol. The results were consistent with the report that resveratrol was metabolized *in vitro* by recombinant human cytochrome P450 isozyme CYP 1B1 to form piceatannol, where the identity was confirmed by GC/MS after derivatisation (5).

Both substrate and metabolite can be easily detected by electrochemical oxidation. The multi-channel electrochemical detector allows for simultaneous application of different oxidation potentials on four separate electrodes. Therefore, the time needed to construct a hydrodynamic voltammogram (HDV) can be reduced substantially. The normalized response of substrate and metabolite at the individual electrodes was plotted against oxidation potential and is presented in ***F5***. Based on the HDV of standard piceatannol, the four channels were set at oxidation potentials of +400, +500, +600 and +700 mV for all subsequent determinations carried out in the study to obtain enzyme kinetic parameters for resveratrol metabolism. Liquid chromatography with multi-channel electrochemical detection (LC/EC) provides a powerful tool for the identification of metabolite. ***F6*** shows LC/EC chromatograms of a piceatannol standard and an incubation sample of resveratrol. Peak height ratios between different oxidation potentials of a standard can be used for peak identification of the retention-equivalent peak in the metabolism incubation sample. Such peak height ratios of standard and sample from ***F6*** have similar values. The close correlation confirms the peak assignment in the incubation sample.

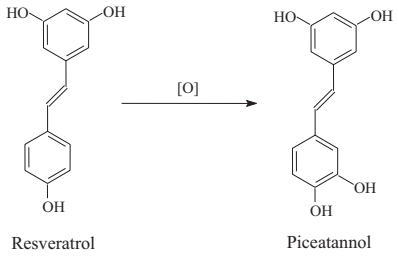
The Lineweaver-Burk plot of initial velocity vs. substrate concentration (concentration of piceatannol produced vs. concentration of resveratrol in reaction mix) is presented in ***F7***. The K_m and V_{max} for resveratrol in the microsomal incubation were determined to be 15.64 ± 2.20 µM (n = 3) and 28.13 ± 3.39 pmole/min/mg protein (n = 3), respectively, under the reported conditions.

Conclusion

An LC/MSMS method has been developed for identification and determination of resveratrol and its *in vitro* microsomal metabolite piceatannol. Identity of the metabolite piceatannol was confirmed by full scan MSMS experiments. The current study

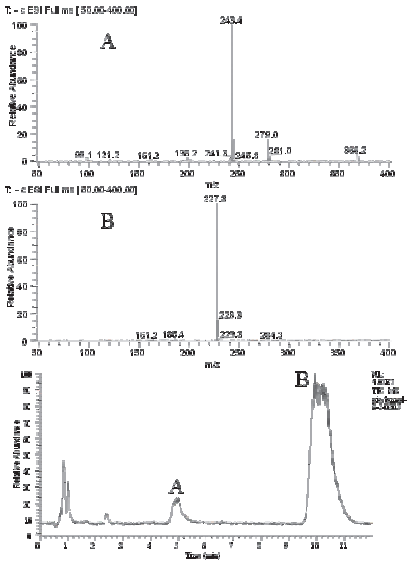
F1.

Structures of resveratrol and piceatannol.



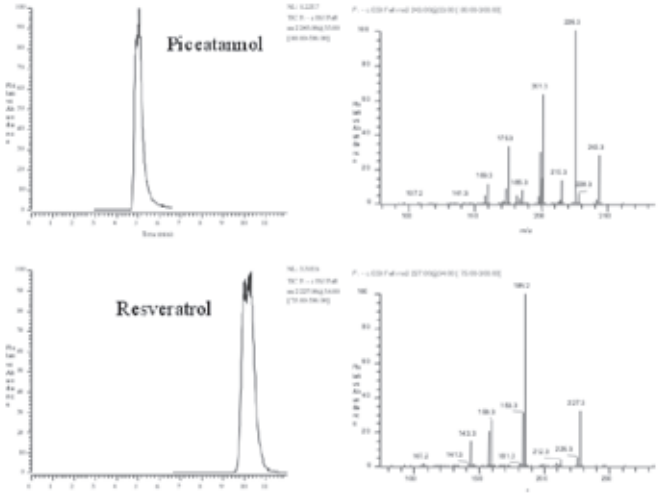
F2.

Mass spectra and representative LC/MS chromatogram of metabolite (A) and resveratrol (B).



F3.

Chromatograms and full scan MSMS spectra of standard mixture of piceatannol and resveratrol.



has demonstrated that LC/EC and LC/MSMS methods may be effective in the study of the *in vivo* metabolism of resveratrol.

Acknowledgements

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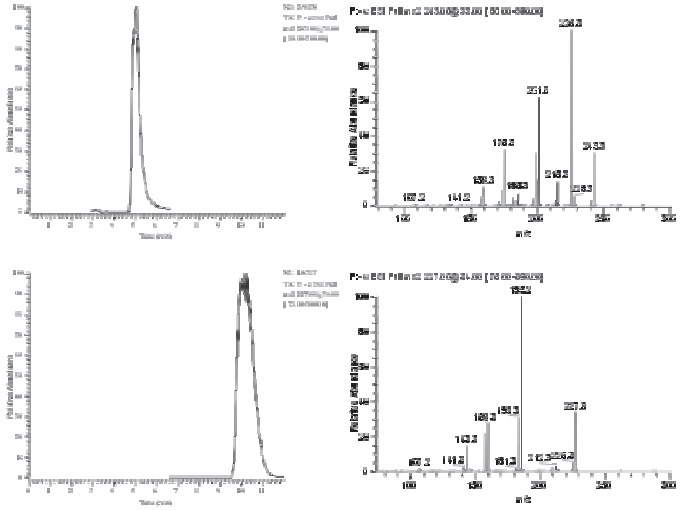
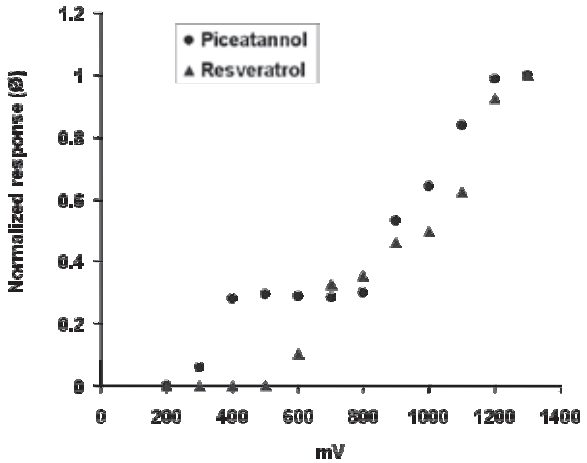
F4.
Chromatograms and full scan MSMS spectra of a rat liver microsomal incubation sample of resveratrol.

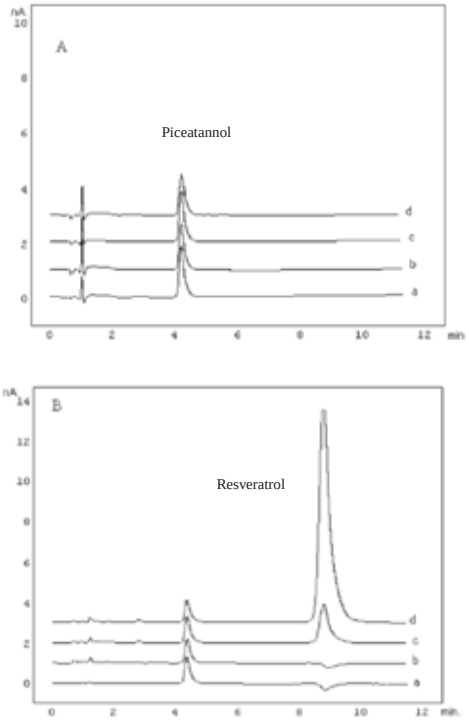


F5.
Hydrodynamic voltammograms (HDV) of resveratrol and piceatannol.



F6.

Chromatograms of standard piceatannol (A) and incubation sample of resveratrol (B). Applied potentials were a) +400, b) +500, c) +600 and d) +700 mV vs Ag/AgCl.



F7.

Lineweaver-Burk plot for resveratrol. Resveratrol (1-50 µM) was incubated with rat liver microsomes (1.0 mg/mL) for 15 min at 37°C.