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Metabolism of N_ω-methylserotonin, a serotonergic constituent of black cohosh (*Cimicifuga racemosa*, L.Nutt.), by human liver microsomes

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Keywords

N-methylserotonin; black cohosh; metabolism; mass spectrometry

Introduction

The roots/rhizomes of black cohosh (*Cimicifuga racemosa* L. (Nutt.) (syn. *Actaea racemosa* L.) have been used traditionally by Native Americans to treat colds and rheumatism as well as for alleviating menopausal symptoms such as hot flashes (McKenna *et al.*, 2001). Because of health risks associated with hormone replacement therapy (Rossouw *et al.*, 2002), black cohosh preparations are popular among women seeking alternative treatments for menopausal complaints (Mahady *et al.*, 2003). Extensive preclinical and clinical investigations have provided conflicting evidence regarding the efficacy of black cohosh for relieving hot flashes (Borrelli and Ernst, 2008). While early studies suggested that black cohosh extracts were effective in reducing the frequency and intensity of hot flashes among perimenopausal and postmenopausal women (Frei-Kleiner *et al.*, 2005; Kronenberg and Fugh-Berman, 2002; Osmers *et al.*, 2005; Wuttke *et al.*, 2003), recent trials including double-blind placebo-controlled studies demonstrated no vasomotor symptom benefits (Geller *et al.*, 2009; Jacobson *et al.*, 2001; Liske *et al.*, 2002; Newton *et al.*, 2006).

The mechanism(s) of action of black cohosh is still a matter of intense research. Several *in vitro* and *in vivo* studies have established that black cohosh extracts are not estrogenic (Liske *et al.*, 2002; Liu *et al.*, 2001; Onorato and Henion, 2001; Overk *et al.*, 2008; Ruhlen *et al.*, 2007). A serotonergic hypothesis was put forward by our group after it was found that crude extracts of black cohosh acted as partial agonists of serotonin 5-HT_{1A} and 5-HT₇ receptors (Burdette *et al.*, 2003). Subsequent bioassay-guided fractionation studies identified N_ω-methylserotonin (N_ω-MeSer) as a potent agonist of the serotonin receptors 5-HT_{1A} and 5-HT₇ with IC₅₀ values of 2 and 0.02 nM, respectively (Powell *et al.*, 2008). This compound also exhibited potent selective serotonin reuptake inhibition activity with an IC₅₀ value of

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490 nM. Because of the significance of N_{ω} -MeSer as a potential active ingredient in black cohosh, it is of interest to understand its human metabolism, bioavailability and pharmacokinetics. In this study, we investigated the *in vitro* metabolism of N_{ω} -MeSer using pooled human liver microsomes. The metabolites were identified by using liquid chromatography-tandem mass spectrometry (LC-MS) and by comparison with authentic standards.

Experimental

Chemicals and reagents

HPLC-grade (Optima) solvents were purchased from Thermo Fisher (Hanover Park, IL). 5-Hydroxytryptophol was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 5-Hydroxyindole acetaldehyde and its stable bisulfite adduct were kind gifts from Dr. Jonathan Doorn (University of Iowa, College of Pharmacy). N_{ω} -MeSer oxalate and 5-hydroxyindoleacetic acid and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Human liver microsomes

Human liver microsomes pooled from 10 women donors (protein content 20 mg/ml; total cytochrome P450 content: 370 pmolP450/mg protein based on the method of Omura and Sato (Omura and Sato, 1964)) were obtained from Gentest (Woburn, MA). Human monoamine oxidase (MAO) SupersomesTM were purchased from Gentest (Woburn, MA). MAO content was measured using kunyramine as the substrate and was 92 and 41 nmol/min/mg for MAO-A and MAO-B, respectively.

N_{ω} -MeSer metabolism by liver microsomes

A typical incubation mixture (0.2 ml) contained 0.5 mg/ml liver microsomes, 10 μ M N_{ω} -MeSer, and 1 mM NADPH in 100 mM potassium phosphate buffer, pH 7.4. The reactions were initiated by the addition of NADPH after a 2-min preincubation of the substrate and the microsomal proteins. Incubations were carried out for 30 min at 37°C. The reactions were stopped by chilling the mixture on ice followed by addition of 0.4 ml of cold acetonitrile to precipitate proteins. Samples were centrifuged, and the supernatant was evaporated to dryness under nitrogen. The residue was reconstituted in the mobile phase prior to LC-MS analysis. Control incubations were carried out without microsomal protein or without NADPH. To prevent degradation of the aldehyde metabolite (see below) some reaction mixtures were supplemented with 1 mM sodium bisulfite.

LC-MS analysis of metabolites

Reversed phase HPLC separations were carried out using Waters (Milford, MA) Atlantis T3 2.1 $\times$ 100 mm C_{18} column (5 μ m particle size) connected to a Waters 2690 solvent delivery system. Metabolites were separated using a gradient system consisting of 0.1% formic acid in water (solvent A) and methanol (solvent B) as follows: 5-16%B over 10 min, then 16-70%B over 10 min followed by an isocratic hold at 70%B for another 5 min. The flow rate was 0.2 ml/min. The column was thermostated at 25°C. The eluent from the column was introduced into a Waters SYNAPT hybrid quadrupole/time-of-flight mass spectrometer

operated in positive ion electrospray mode. The resolving power was set at 10,000 full width at half maximum. For accurate mass measurements, Leu-enkephalin was introduced as a standard via a separate sprayer. The mass accuracy was within 5 ppm unless noted otherwise. Tandem mass spectra were acquired using collision-induced dissociation at a collision energy of 20 eV in the trap region using argon as the collision gas.

Kinetic studies

To determine kinetic constants for conversion of serotonin and N_{ω} -MeSer into 5-hydroxyindol acetaldehyde, the reaction mixture (0.2 ml) contained 0.1 mg/ml MAO-A microsomal protein and appropriate amounts of serotonin or N_{ω} -MeSer (50-2000 μ M) in 100 mM potassium phosphate buffer (pH 7.4). Reactions were carried out for 15 min at 37°C and stopped by adding equal volumes of acetonitrile containing internal standard (which was serotonin when N_{ω} -MeSer was substrate and N_{ω} -MeSer when serotonin was the substrate). Under these conditions, formation of the product was linear with respect to time and protein concentration. Quantitation of the product 5-hydroxyindol acetaldehyde was carried out using HPLC with UV detection at 280 nm. The separation of the product was carried out using the same Waters column described above, except that a linear gradient from water (solvent A) to methanol (solvent B) was as follows: 5-95%B over 10 min followed by an isocratic hold at 95%B for 2 min. The flow rate was 0.3 ml/min and the column temperature was 30°C. Calibration curves were prepared by diluting authentic standard with buffer immediately prior to analysis.

Results

The total ion and computer-reconstructed ion mass chromatograms from the positive ion electrospray LC-MS analysis of incubations of 10 μ M N_{ω} -MeSer with pooled human liver microsomes are shown in Figure 1. Analysis of chromatograms revealed that metabolism of N_{ω} -MeSer occurred even in the absence of NADPH, indicating that an enzyme(s) other than the cytochrome P450s was responsible for the metabolism of this compound. Inspection of the chromatograms revealed a presence of a metabolite (M1) with the molecular formula of $C_{10}H_9NO_2$ (-2.5 ppm). The product ion tandem mass spectrum of M1 (m/z 176) is shown in Figure 2. By analogy with the known metabolism of serotonin, formation of 5-hydroxyindole acetaldehyde was suspected. In the presence of sodium bisulfite, a new peak corresponding to the stable adduct was observed that had identical retention time with the authentic standard of 5-hydroxyindole acetaldehyde bisulfite adduct (see Supplemental Figure S1). Comparison of retention times and tandem mass spectrometric fragmentation patterns with an authentic standard confirmed identification of M1 as 5-hydroxyindol acetaldehyde.

Since 5-hydroxyindol acetaldehyde was detected in the absence of NADPH, involvement of microsomal MAOs was tested by carrying out incubation in the presence of MAO inhibitor clorgyline. Clorgyline completely inhibited formation of 5-hydroxyindol acetaldehyde (Figure 1) indicating that MAO was responsible for its formation. To determine which MAO isoform was responsible for the production of M1, incubations with human recombinant MAO-A and MAO-B were carried out. As shown in Figure 3, only MAO-A catalyzed

formation of 5-hydroxyindole acetaldehyde. The kinetic parameters for this reaction were very similar to those for serotonin (Table 1), indicating that serotonin and N_{ω} -MeSer are equally good substrates for MAO-A.

Besides M1, there were two other major metabolites (M2 and M3) present in incubation mixtures both in the presence and absence of NADPH (Figure 1). Metabolite M2 (m/z 178) had molecular formula of $C_{10}H_{11}NO_2$ (- 4.5 ppm), while M3 (m/z 192) had a molecular formula of $C_{10}H_9NO_3$ (+ 2.1 ppm). Upon fragmentation, M2 and M3 produced the tandem mass spectra shown in Figure 2. Based on these data and comparison with authentic standards, M2 was identified as 5-hydroxytryptophol, while M3 was identified as 5-hydroxyindole acetic acid. Both M2 and M3 were formed from the primary metabolite M1, but the ratio between the two varied depending on the presence of NADPH in the reaction mixture (Figure 1). When NADPH was present, the alcohol metabolite M2 was predominantly formed while in the absence of NADPH, chemical disproportionation resulted in similar amounts of M2 and M3. In the presence of clorgyline, no M2 and M3 were detected further confirming that they originate from a precursor metabolite M1 formed by the action of MAO-A.

When MAO-A activity was inhibited by clorgyline, three minor oxidation products were detected in incubations containing NADPH indicating that cytochrome P450s are capable of oxidizing N_{ω} -MeSer. Tandem mass spectra of these metabolites are shown in Figure 4. The presence of an ion of m/z 176 indicated that oxidation did not occur on the nitrogen atom. However, beyond this basic information, tandem mass spectra alone were not sufficient to identify these metabolites.

Discussion

Our recent investigations of the serotonergic constituents of black cohosh revealed that N_{ω} -MeSer was the main component responsible for *in vitro* serotonergic activity of this plant (Powell *et al.*, 2008). Even though the amount of N_{ω} -MeSer in black cohosh is small, it is one of the few confirmed sources of human exposure to this potent serotonergic ligand. It has also been found in several species of *Citrus* plants and in glucosidated form, in immature seeds of Japanese pepper (*Zanthoxylum piperitum*) (Servillo *et al.*, 2013; Yanase *et al.*, 2010). Our data from these studies with human microsomes indicate that metabolism of N_{ω} -MeSer is essentially the same as that of serotonin. The major metabolic pathway of N_{ω} -MeSer is MAO-A catalyzed conversion into 5-hydroxyindol acetaldehyde which can be further oxidized to 5-hydroxyindole acetic acid or reduced into 5-hydroxytryptophol. Since 5-hydroxyindol acetaldehyde is an unstable compound, it can readily disproportionate into the corresponding acid and alcohol. 5-hydroxyindole acetic acid is also an unstable compound, thus the actual ratio of acid vs. alcohol metabolites obtained in incubations without NADPH will depend on particular experimental conditions used for sample processing such as drying time, exposure to light, oxygen or heat. When NADPH was present, microsomal aldehyde reductase predominantly converted 5-hydroxyindol acetaldehyde into the alcohol (see Figure 5). In case of serotonin, the acid metabolite predominates *in vivo*, however the ratio of acid versus alcohol metabolite can be influenced by a number of genetic, dietary and environmental factors, such as for example alcohol

consumption (Helander *et al.*, 1994; Johnson *et al.*, 2005; Kema *et al.*, 2000). Given the similarities of N_{ω} -MeSer metabolism this is likely to hold true for this compound as well, thus the ratios obtained in *in vitro* incubations are of little relevance for the *in vivo* situation. The similar metabolic fate of N_{ω} -MeSer and serotonin would make it difficult to investigate its metabolism *in vivo* as it would be impossible to distinguish whether urinary 5-hydroxyindole acetic acid and 5-hydroxytryptophol originate from serotonin or N_{ω} -MeSer.

In situations where MAO-A is inhibited, such as concomitant consumption of MAO-A inhibitors either present in the extract itself or as a part of therapy, the metabolism of N_{ω} -MeSer can switch to a cytochrome P450 mediated pathway. This was, however, only a minor metabolic route suggesting that inhibition of MAO-A might be a strategy to improve the bioavailability of N_{ω} -MeSer.

Given that MAO-A is a ubiquitous enzyme widely distributed along the absorption pathways and in the blood-brain barrier, the oral bioavailability and CNS penetration of N_{ω} -MeSer is predicted to be low. Given the small amount of this compound in black cohosh extracts (Powell *et al.*, 2008), it is likely that *in vivo* activities of black cohosh are not mediated by this compound. This is consistent with what is known about other biogenic amines such as serotonin and bufotenin, both of which are not orally active. However, if MAO-A inhibitors should occur in black cohosh extracts or other botanical supplements being consumed at the same time, they might minimize first-pass metabolism of this compound and improve its bioavailability. Then, additional studies would be warranted to address this issue.

Conclusion

This study demonstrated that metabolism of N_{ω} -MeSer is qualitatively and quantitatively similar to that of serotonin. Given that MAO-A is the major enzyme involved in the metabolism of N_{ω} -MeSer and is ubiquitous along its absorption routes, the bioavailability of N_{ω} -MeSer is likely to be low. Combined with the low abundance of this compound in black cohosh extracts, it is likely that N_{ω} -MeSer is not responsible for the observed biological activities of black cohosh.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Positive ion electrospray total ion chromatograms and computer-reconstructed mass chromatograms from incubation of 10 μ M N_ω -methylserotonin with human liver microsomes. The peak labeled with * is an impurity in the microsomal preparation.

Figure 2.
Product ion tandem mass spectra of N_ω -methylserotonin and its major metabolites.

Figure 3.
LC-UV chromatograms from incubations of N_ω-methylserotonin with human recombinant MAOs showing that N_ω-methylserotonin is a substrate for MAO-A but not MAO-B.

Figure 4.

Product ion tandem mass spectra of minor CYP450 mediated metabolites of N_ω -methylserotonin. These minor metabolites were only formed when MAO-A was inhibited by clorgyline. The limited information provided by these tandem mass spectra did not allow for the identification of these products.

Figure 5.

Summary of metabolic pathways of N_ω-methylserotonin in human liver microsomes. These are similar to those observed for endogenous neurotransmitter serotonin. Abbreviations: ALR=aldehyde reductase; ALDH=aldehyde dehydrogenase.

Table 1
Kinetic parameters for formation of 5-hydroxyindol acetaldehyde by human recombinant MAO-A

Substrate	K_m	V_{max}	V_{max}/K_m
	μM	$pmol/min/pmol\ MAO-A$	
N ₆ -methylserotonin	123 ± 10^a	50.9 ± 4.2^a	0.414
Serotonin	191 ± 15	82.2 ± 6.9	0.430

^aData represent mean $\pm$ SD (N=3)