Human Consumption of Methyleugenol and Its Elimination from Serum

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Under a mandate from the U.S. Congress, the National Toxicology Program (NTP) of the U.S. Department of Health and Human Services conducts animal bioassays for carcinogenicity of potentially toxic chemicals to which the U.S. population might be exposed. Methyleugenol, a natural as well as synthesized substance, was nominated for study because it is structurally similar to safrole, a known animal carcinogen. Methyleugenol was found to be a very potent multisite carcinogen in male and female F344/N rats and B6C3F$_1$ mice at all doses tested in 2-year NTP bioassays using gavage dosing. For this reason, human toxicokinetic studies were added to the traditional NTP protocol. A commercial brand of gingersnaps was found by chemists at the Centers for Disease Control and Prevention to contain a relatively high concentration of methyleugenol. After thorough scientific and clinical review, and approval by a National Institutes of Health institutional review board for the protection of human subjects, a study was conducted with nine healthy adult male and female human volunteers. The volunteers were given 12 gingersnaps for breakfast. Blood was drawn immediately before the meal and at 15, 30, 60, and 120 min afterward. The mean ± SD fasting level of methyleugenol in serum was 16.2 ± 4.0 pg/g wet weight. Peak blood levels were found at 15 min (mean ± SD, 53.9 ± 7.3 pg/g wet weight), followed by a rapid decline; the half-life of elimination was about 90 min. The peak levels were within the range of methyleugenol blood levels in the U.S. population, as measured concurrently in a subset of nonfasting participants in the Third National Health and Nutrition Examination Survey (NHANES III). ***Key words:*** **4-allyl-1,2-dimethoxybenzene, carcinogen, dose, food, human, ingestion, kinetics, methyleugenol, serum levels, urine.** ***Environ Health Perspect*** **112:678–680 (2004). doi:10.1289/ehp.6766 available via** ***http://dx.doi.org/*** [Online 2 February 2004]

Methyleugenol, or 4-allyl-1,2-dimethoxybenzene, occurs naturally in clove oil, nutmeg, anise, allspice, cinnamon bark, walnuts, and several exotic herbs and spices. Either synthesized or of natural origin, methyleugenol is in common use as a flavoring agent in candy, cookies such as gingersnaps, ice cream, pies such as pumpkin pie, puddings, cola soft drinks, bubble gum, French toast, eggnog topped with nutmeg, patés and terrines, tomato ketchup and relish, sweet chili sauce, apple butter, chutney, anise biscotti, mincemeat, and gingerbread (Burdock 1995; Leung 1980). Methyleugenol is also used as a fragrance in some perfumes and toiletries at concentrations of 0.002–0.3%. U.S. annual production of synthesized methyleugenol is about 25,000 pounds (SRI International 1990).

Because of its widespread use and structural resemblance to the known carcinogens safrole, isosafrole, and estragole, methyleugenol was nominated for toxicologic characterization and testing by the National Toxicology Program (NTP) (Miller et al. 1983; NTP 1998). In these 2-year gavage studies, significant dose-related increases in neoplastic lesions of the liver were found in both male and female F344/N rats and B6C3F$_1$ mice. In addition, increases of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma and fibrosarcoma occurred in male rats, and neuroendocrine tumors of the glandular stomach occurred in male mice and in male and female rats. Also, methyleugenol is shown to possess cytotoxicity and genotoxicity in rat hepatocytes (Burkey et al. 2000). These findings constituted "clear evidence of carcinogenic activity," the highest category of evidence used by the NTP for animal bioassays (NTP 1998).

The highest concentration of methyleugenol found among food products subjected to preliminary semiquantitative analyses by the National Center for Environmental Health of the Centers for Disease Control and Prevention (CDC) was approximately 3.3 µg/g methyleugenol in one brand of gingersnaps. Lower concentrations were found (in decreasing order) in other brands of gingersnaps, cinnamon-flavored oatmeal, vinaigrette salad dressing, cinnamon-flavored mints, a "red" brand of chewing gum, cake doughnuts, and cola beverages. The analytical methods used for the food have not been validated for accuracy, reproducibility, or detection limits. In 20 other brands of gingersnaps and other cookies, doughnuts, colas, and foods with cinnamon, nutmeg, or ginger flavoring, methyleugenol either was not detected or was found at concentrations < 0.05 µg/g. Methyleugenol has also been measured in tobacco from U.S. cigarettes (0.003–0.86 µg/g) and at higher concentrations in flavored bidi cigarettes (0.49–61 µg/g) (Stanfill et al. 2003).

In mice and rats, methyleugenol is metabolized very rapidly, and the metabolites are found in urine within hours (NTP 1998). Metabolism of the compound in humans has not previously been studied. To the best of our knowledge, no previous data exist on the levels of methyleugenol or its metabolites in urine of the U.S. general population, and only one recently published study exists on blood levels of methyleugenol (Barr et al. 2000).

The purpose of this collaborative study was to determine the levels of methyleugenol in human blood at certain times before and after eating a common commercial food product, gingersnap cookies, known to contain the compound.

Materials and Methods

Study subjects. Twenty adults living in the Raleigh–Durham area of North Carolina volunteered for the study in response to an announcement that included a nominal payment in return for time spent for the experiment. None were affiliated with the National Institute of Environmental Health Sciences (NIEHS). The study protocol, including informed consent procedure, was approved by the appropriate institutional review boards for the protection of human subjects at the National Institutes of Health.

Volunteers were screened on the basis of a standard occupational/environmental medical history from Duke Medical Center (Durham,

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NC) (Duke Occupational Health Service 1966). Pregnant women and persons with a diagnosed illness were excluded, as were persons taking any medicine for treatment of illness, with the exception of aspirin, acetaminophen, allergy medications, and oral contraceptives. Five male and four female volunteers met the inclusion criteria and agreed to participate.

Feeding study. The volunteers were instructed to fast from midnight until reporting to the NIEHS health station for the study at 0730 hr. Each person arrived with a completed food survey listing all foods consumed after dinner the previous evening. There were no dietary restrictions. Height, weight, temperature, and blood pressure were recorded, followed by an initial blood draw.

Volunteers were then served a breakfast known to contain methyleugenol, consisting of the brand of gingersnaps found on initial analysis to contain approximately 3.3 µg methyleugenol per gram, or 18 µg/cookie. The gingersnaps were purchased locally from a large supermarket chain. Orange juice was provided as a beverage. Each subject's meal consisted of 12 gingersnaps, containing approximately 216 µg methyleugenol. This was the largest practical dosing felt to be representative of a moderate dietary intake in average adult volunteers ingesting commercial food containing methyleugenol.

Specimen collection. Blood was collected at 15, 30, 60, and 120 min after the meal of gingersnaps. The times were based on a model of the anticipated rates of absorption, distribution, and elimination of methyleugenol in humans developed before contracting this study (Blumenthal G, personal communication). Blood samples were centrifuged and the cell layer discarded. Serum for future study were frozen after collection and sent to CDC frozen on dry ice.

Methyleugenol analysis. Methyleugenol analyses were performed on a 4-mL aliquot of serum spiked with a $^{13}C_3$-labeled methyleugenol internal standard (Barr et al. 2000). The serum was filtered to 0.2 µL and denatured with 50% formic acid, and then aspirated through a C_{18} solid-phase extraction cartridge. The cartridge was washed with water and eluted with methylene chloride. The eluate was cleaned up on a silica cartridge with 1 g anhydrous sodium sulfate and then concentrated to 10 µL after addition of a keeper solvent and recovery standard. A 2-µL sample was analyzed by gas chromatography–high-resolution mass spectrometry in the single-ion monitoring mode at 10,000 resolution. Two ions were monitored for methyleugenol (*m/z* 178.0994, which is M^+, and *m/z* 163.0759, which is $M\text{-}CH_3^+$) and one ion each for the labeled internal standard (*m/z* 181.1094) and recovery standard [*m/z* 164.0473; 3′,4′-(methylenedioxy)-acetophenone]. The 178.0994 ion was used for quantification and the 163.0759 for confirmation. The limit of detection of the method was 3.1 pg/g, and the relative SD (RSD) ranged from 8.9% to 21%. The higher RSDs were seen at the low concentration end of the linear range. Extensive checks for quality control and quality assurance were incorporated into the method (Barr et al 2000).

Results

Subjects. Age, sex, height, and weight of subjects are shown in Table 1. All were healthy residents of North Carolina living in or near Durham, Cary, or Research Triangle Park.

Serum levels. Measured levels of methyleugenol as picograms per gram (parts per trillion) in serum are presented in Table 2 and as nanograms per gram (parts per billion), lipid basis, in Table 3. The time course of median wet weight serum levels is represented graphically in Figure 1. The median fasting level of methyleugenol in serum was 13 pg/g wet weight (range, < 3.1–37 pg/g). The median serum level at 15 min peaked at 54 pg/g wet weight (range, 25–100 pg/g). The median level declined to 40 pg/g (range, 26–99 pg/g) at 30 min, to 29 pg/g (range, 13–94 pg/g) at 1 hr, and to 20 pg/g (range, 15–61 pg/g) at 2 hr.

Discussion

Results for methyleugenol levels in serum from the human clinical study, in which subjects ate gingersnaps, were consistent with expectations (based on elimination rates in male and female F344/N rats and $B6C3F_1$ mice) that methyleugenol blood levels in

Table 1. Methyleugenol subject profiles.

Subject	Sex	Age (years)	Weight (lbs)	Height (inches)
A	Male	20	175	71
C	Female	20	109	63
D	Female	47	116	63
E	Male	37	176	74
F	Female	27	235	68
H	Male	50	189	70
I	Male	21	164	74
J	Female	36	133	63
K	Male	37	146	70

Table 2. Serum levels [pg/g (ppt) wet weight] of methyleugenol in nine human volunteers at five time points before and after dosing.

	Subject										
Time	A	C	D	E	F	H	I	J	K	Mean	Median
Prefeed	16	30	25	37[a]	9.5	13	5.2	8.8	ND[b,c]	16.2	13
15 min	58	100[a]	60	43	40	69	54	25[b]	36	53.9	54
30 min	38	99[a]	42	26	26[b]	40	40	47	28	42.9	40
1 hr	46	94[a]	41	23	13[b]	23	28	29	36	37.0	29
2 hr	20	61[a]	37	15[b]	20	20	15	19	20	25.2	20

ND, not detected.
[a]Maximum value for row. [b]Minimum value for row. [c]Half the limit of detection (1.55 pg/g) was used as the serum value in calculating the mean.

Table 3. Serum levels [ng/g (ppb) lipid basis] of methyleugenol in nine human volunteers at five time points before and after dosing.

	Subject										
Time	A	C	D	E	F	H	I	J	K	Mean	Median
Prefeed	2.5	5.2	4	5.7[a]	1.1	2.2	1.3	2	ND[b,c]	2.7	2.2
15 min	9.3	17[a]	9.7	6.6	4.4[b]	12	13	5.4	5.4	9.2	9.3
30 min	6.1	17[a]	6.9	4.0	3.2[b]	6.8	9.6	10	4.7	7.6	6.8
1 hr	7.4	16[a]	6.3	3.5	1.5[b]	4.2	6.9	6.5	5.7	6.4	6.3
2 hr	3.4	10[a]	5.6	2.2[b]	2.3	3.5	2.8	4.3	3.7	4.2	3.5

ND, not detected.
[a]Maximum value for row. [b]Minimum value for row. [c]In calculation of the mean, 0.3 ng/g was used as the lipid-based level.

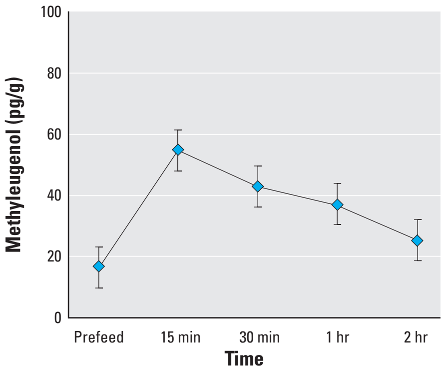

Figure 1. Median of methyleugenol serum concentrations for nine subjects [pg/g (ppt) wet weight]. Error bars indicate SE.

human adults would peak rapidly after a meal and have a half-life of elimination of about 90 min. The mean ± SD methyleugenol plasma concentrations (wet weight) at the lowest dose (37 mg/kg) 15 min after gavage exposure were 573 ± 228 ng/g (ppb) for male rats and 652 ng/g (two samples, no SD) for female rats. In mice, the mean ± SD concentrations in plasma (wet weight) for the lowest exposure group (37 mg/kg) 10 min after exposure were 417 ± 128 ng/g for males and 681 ± 50 ng/g for females (NTP 1998). At these doses, the increased liver cancer risks were 17.5%, 17.1%, 34%, and 52.2% in male and female rats and male and female mice, respectively. The human plasma concentrations were roughly 10,000 times smaller, with an average concentration of 0.0539 ± 0.0083 ng/g. The average weight for humans was 68.3 kg yielding a dosage of 3.16 µg/kg, which is approximately 10,000 times smaller than the dose given the rodents.

For humans, the estimated half-life of methyleugenol was approximately 100 min (95% confidence interval, 1–6 hr).

In a concurrent study of 213 nonfasting subjects in the third National Health and Nutrition Examination Survey (NHANES III, 1988–1994), the range of serum levels of methyleugenol was < 3.1–390 pg/g, with a median of 16 pg/g (National Center for Health Statistics 1994). In 16 subjects, serum levels of methyleugenol exceeded 54 pg/g, the median peak level observed in the clinical study. Four NHANES subjects had serum levels exceeding 100 pg/g, the highest peak level measured in this clinical experiment. The maximum concentration found, 390 pg/g, is nearly four times the highest concentration found in this clinical study but still nearly 2,000 times less than peak levels observed at the lowest dose used in the NTP rodent studies (Barr et al. 2000).

Methyleugenol has been proposed to be metabolized extensively by various cytochrome P450 isozymes, with metabolism rates by human liver microsomal preparations varying more than 37-fold (Gardner et al. 1997). Therefore, this broad range of concentrations found in the NHANES sample (from below the limit of detection to 300 pg/g) may represent a combination of differences in metabolic rates, time since last meal, and food consumed at last meal.

The finding of methyleugenol in the blood of the general U.S. population at levels higher than the peak levels obtained in the clinical study was unexpected, and the significance of these levels with respect to health consequences remains to be determined. The information from these studies has been shared with the U.S. Food and Drug Administration. The reasons for an elevated level in a substantial number of adults in the general U.S. population require further investigation, as does the risk of cancer and other health effects associated with background levels of exposure to methyleugenol.

References

Barr DB, Barr JR, Bailey SL, Lapeza CR Jr, Beeson MD, Caudill SP, et al. 2000. Levels of methyleugenol in a subset of adults in the general U.S. population as determined by high resolution mass spectrometry. Environ Health Perspect 108:323–328.

Burkey JL, Sauer JM, McQueen CA, Sipes IG. 2000. Cytotoxicity and genotoxicity of methyleugenol and related congeners—a mechanism of activation for methyleugenol. Mutat Res 453:25–33.

Burdock GA, ed. 1995. Fenaroli's Handbook of Flavor Ingredients, Vols. 1 and 2. Boca Raton, FL:CRC Press.

Duke Occupational Health Service. 1996. Comprehensive Health History Form. Durham, NC:Duke Occupational Health Service.

Gardner I, Wakazono H, Bergin P, de Waziers I, Beaune P, Kenna JG, et al. 1997. Cytochrome P450 mediated bioactivation of methyleugenol to 1′-hydroxymethyleugenol in Fischer 344 rat and human liver microsomes. Carcinogenesis 18:1775–1783.

Leung AY, ed. 1980. Encyclopedia of Common Natural Ingredients. New York:John Wiley and Sons.

Miller EC, Swanson AB, Phillips DH, Fletcher AL, Miller JA. 1983. Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res 43:1124–1134.

National Center for Health Statistics. 1994. Plan and Operation of the Third National Health and Nutrition Examination Survey, 1988–94. Series 1: Program and Collection Procedures No. 32. Available: http://www.cdc.gov/nchs/data/series/sr_01/sr01_032.pdf [accessed 18 March 2004].

NTP. 1998. Toxicology and Carcinogenesis Studies of Methyleugenol (CAS No. 93-15-12) in F344/N Rats and B6C3F$_1$ Mice. NTP Technical Report 491. Research Triangle Park, NC:National Toxicology Program.

SRI International. 1990. Directory of Chemical Producers, United States of America. Menlo Park, CA:SRI International.

Stanfill SB, Calafat AM, Brown CR, Polzin GM, Chiang JM, Watson CH, et al. 2003. Concentrations of nine alkenylbenzenes, coumarin, piperonal and pulegone in Indian bidi cigarette tobacco. Food Chem Toxicol 41(2):303–317.