Absorption, Distribution, and Biliary Excretion of Cafestol, a Potent Cholesterol-Elevating Compound in Unfiltered Coffees, in Mice


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ABSTRACT:
Cafestol is a diterpene present in unfiltered coffees. It is the most potent cholesterol-elevating compound present in the human diet. However, the precise mechanisms underlying this effect are still unclear. In contrast, cafestol is also known as a hepatoprotective compound, which is likely to be related to the induction of glutathione biosynthesis and conjugation. In the present study, we investigated whole-body distribution, biliary excretion, and portal bioavailability of cafestol in mice. First, dissection was used to study distribution. Five hours after an oral dose with 3H-labeled cafestol, most activity was found in small intestine, liver, and bile. These results were confirmed by quantitative whole-body autoradiography in a time course study, which also showed elimination of all radioactivity within 48 h after administration. Next, radiolabeled cafestol was dosed intravenously to bile duct-cannulated mice. Five hours after the dose 20% of the radioactivity was found in bile. Bile contained several metabolites but no parent compound. After intestinal administration of radioactive cafestol to portal vein-cannulated mice, cafestol was shown to be rapidly absorbed into the portal vein as the parent compound, a glucuronide, and an unidentified metabolite. From the presence of a glucuronide in bile that can be deconjugated by a bacterial enzyme and the prolonged absorption of parent compound from the gastrointestinal tract, we hypothesized that cafestol undergoes enterohepatic cycling. Together with our earlier observation that epoxidation of the furan ring occurs in liver, these findings merit further research on the process of accumulation of this coffee ingredient in liver and intestinal tract.

In addition to its deleterious effects on cholesterol levels and liver enzymes, cafestol has also been identified as an antimutagenic compound (Cavin et al., 2002; Huber et al., 2002a,b, 2003, 2004; Huber and Parzefall 2005). This potential beneficial effect was shown to be related to an induction of glutathione biosynthesis and conjugation and a decreased activity of cytochrome P450 enzymes involved in the bioactivation of some procarcinogens (Cavin et al., 2003; Huber et al., 2004, 2008; Lee et al., 2007). We recently demonstrated that cafestol is able to induce an electrophile-response element (EpRE) in vitro after metabolic activation with an S9 mix obtained from the livers of male Sprague-Dawley rats treated with Aroclor-1254 (van Cruchten et al., 2009). The EpRE is a regulatory sequence mediating the coordinated transcriptional activation of genes associated with phase 2 biotransformation, protection against oxidative stress, and other cancer-chemoprotective mechanisms (Kobayashi et al., 2006). The key regulator of EpRE-mediated gene expression is the transcription factor nuclear factor erythroid 2-related factor (Nrf2) and, to a lesser extent, Nrf1 (Copple et al., 2008). This Nrf2 activation may be related nuclear factor erythroid 2-related factor (Nrf2) and, to a lesser extent, Nrf1 (Copple et al., 2008). This Nrf2 activation may be responsible for the increase in glutathione biosynthesis and conjugation. We hypothesized that metabolites formed via epoxidation of the furan moiety are involved in this induction process. Indeed, we were able to demonstrate the presence of cafestol epoxides and their epoxy-

ABBREVIATIONS: EpRE, electrophile-response element; Nrf, nuclear factor erythroid 2-related factor; HPLC, high-performance liquid chromatography; QWBA, quantitative whole-body autoradiography.
glutathione conjugates in bile of mice dosed with cafestol. In addition, we also found a glucuronide conjugate of cafestol in mouse bile (van Cruchten et al., 2009).

The present studies were undertaken to study body distribution, portal bioavailability, and biliary excretion of cafestol in mice in more detail. The selection of the mouse as a model was based on previous studies showing that among various animal models, the ApoE*3-Leiden transgenic mouse is the only model that responds to cafestol as humans do (Post et al., 2000).

Distribution after oral administration of radiolabeled cafestol was studied by liquid scintillation counting of dissected tissues and by quantitative whole-body autoradiography. After the observation that the distribution of the radiolabel remained largely restricted to the liver and intestinal tract, the question of whether enterohepatic cycling plays a role was addressed. This was first investigated by studying biliary excretion of cafestol and metabolites after intravenous administration to gallbladder-cannulated mice. Finally, portal delivery of cafestol and potential metabolites was assessed after intestinal administration.

Materials and Methods

Chemicals. Chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Solvable (tissue-solubilizing fluid) was purchased from PerkinElmer Life and Analytical Sciences (Groningen, The Netherlands). Methanol was purchased from Mallinckrodt Baker (Deventer, The Netherlands).

Radiolabeling. [3H]Cafestol (1 mCi/mmol) was synthesized by RC Tritic (Teufen, Switzerland). The compound was made by hydrogenation of kahweol (Alexis Laboratories, Sandiego, CA) to cafestol under a tritium gas atmosphere using Pd-SrCO3 in ethanol. Labeling was on the C1 and C2 positions. Before each use, radiochemical purity of [3H]cafestol was verified (99%).

FIG. 1. Structure of cafestol. Labeling was on the C1 and C2 positions. Before each use, radiochemical purity of [3H]cafestol was verified (99%).

Organ Sample Preparation. Samples was determined by scintillation counting. These values were averaged after oral administration in more detail and at more points in time. Mice (eight in total) were dosed with 1.5 mg of unlabeled cafestol in olive oil via oral gavage mixed with a trace amount of [3H]cafestol (1 mCi/kg b.wt.). At different time points (0.5, 1, 2, 4, 8, 12, 24, and 48 h) mice were anesthetized with a mixture of isoflurane and oxygen, and blood was sampled via orbital puncture and puncture of the tail vein. Then mice were euthanized with a mixture of isoflurane and oxygen, fixed, and subsequently frozen in a hexane-dry ice bath. Whole-body autoradiography was performed according to standard procedures (SOLON et al., 2002) as described below. Experiment III was performed to study biliary excretion of cafestol and its metabolites after intravenous administration of labeled cafestol. For this experiment, five mice were fasted overnight. Mice were anesthetized, and subsequently their gall-bladder was cannulated and bile was collected (Kuipers et al., 1996; Klett et al., 2004). To maintain a constant bile flow, mice were infused with a bile acid solution containing tauroursodeoxycholic acid (concentration of 600 nmol/100 g b.wt/min) according to standard procedures (Klett et al., 2004). After 60 min of infusion, bile flow was considered constant based on our previous experience, and cafestol was injected through the tail vein. Every mouse received injections of 200 μl of a mixture containing 12 μg of unlabeled compound and 10 μCi of [3H]cafestol dissolved in ClinOtic 20% in the tail vein. Bile was sampled every 15 min during the 1st h and then every 1/2 h. After 5 h, blood samples were drawn from the systemic circulation by orbital puncture. Then mice were killed and liver, intestine, and kidneys were isolated. Bile and plasma samples were immediately frozen at −80°C. Bile flow was determined gravimetrically, assuming density of 1 g/ml for bile. Total radioactivity in the samples was determined by scintillation counting. These values were averaged for the number of mice. Organ samples were processed as described under Organ Sample Preparation.

In the fourth experiment (experiment IV), the portal vein of C57BL/6J mice that had fasted for 4 h was cannulated. A solution containing cafestol mixed with 60 μCi of [3H]cafestol was administered at a dose of 1.5 mg directly into the duodenum. Portal blood was sampled at different time points: 2, 5, 10, 20, 30, 40, and 50 min. At the end (50 min postdose) a blood sample from the systemic circulation was collected by orbital puncture. Blood samples were centrifuged at 4000g for 10 min, and plasma was stored at −80°C until further HPLC analyses.

Organ Sample Preparation. Organ samples were cut in pieces of approximately 25 mg and incubated overnight at room temperature with 1 ml of Solvable. After incubation, samples were decolorized with 300 μl of hydrogen peroxide (30%) (1000 μl in case of spleen) and counted. Blood samples (30 μl) were centrifuged, and the radioactivity in plasma was determined. Five microliters of bile were diluted with 5 ml of scintillation fluid (Ultima Gold, PerkinElmer Life and Analytical Sciences) and incubated at room temperature overnight in a dark environment. The radioactivity in all samples was measured using a liquid scintillation counter (model 3255, PerkinElmer Life and Analytical Sciences, Waltham, MA), with a quench curve used for correction.

Quantitative Whole-Body Autoradiography. The animals were rapidly frozen by total immersion in a hexane-solid carbon dioxide mixture (approximately −80°C) and retained at −80°C for quantitative whole-body autoradiography. After removal of the whiskers, legs, and tail, each frozen carcass was placed in a block of carboxymethyl cellulose (1% aqueous solution, w/v) and mounted onto the stage of a Leica CM360 cryomicrotome maintained at approximately −20°C. Sagittal sections (normally 30 μm) of each animal were subjected to whole-body autoradiography using procedures based on the work of Ulberg (1977), at five different levels of the animal body (to include as many tissues as possible). Three sections were taken at each level and freeze-dried. One section from each level was selected and, along with an H whole-blood standard curve, was placed in contact with Fuji imaging plates. The imaging plates were placed in light-tight cassettes and allowed to expose for 7 days. After exposure, and under subdued lighting, the sections were removed from the plates, and the plates processed using a Fuji BAS 1500 Bio-image analyzer (Fujiﬁlm, Tokyo, Japan). These analyses were performed by Quotient Bioresearch (Rushden, UK).

HPLC Analysis with Radiochemical Detection of Cafestol and Metabolites. Five microciliors of bile sample were mixed with 45 μl of Milli-Q water.

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This sample was analyzed by a HPLC system equipped with a ChromSpher column (Varian, Middelburg, The Netherlands), 100 × 4.6 mm i.d., packed with 3 μm of Omnisphere C18 material (Varian). The mobile phase was delivered using a system with Gynkotech pumps (Gynkotech, Germering, Germany) at a flow rate of 0.4 ml/min. Solvent A was 5 mM phosphate buffer (pH 6.3) and solvent B was 100% methanol. A linear gradient of 0 min at 10% B to 20 min at 100% B was used to separate the components. Detection was performed using an absorbance detector (500TR series; PerkinElmer Life and Analytical Sciences), run in parallel with an absorbance detector (Spectroflow 757; Kratos Analytical Instruments, Ramsey, MI).

Deconjugation of Cafestol Metabolites. Bile samples (5 μl) were deconjugated by adding 10 μl of Escherichia coli-derived β-glucuronidase solution. This mixture was incubated at 37°C for 1 h. HPLC analysis was performed with these samples as described.

Results

Tissue Distribution of [3H]Cafestol (Metabolites). Five hours after oral administration of [3H]-labeled cafestol, the major amount of the radioactivity was found in the intestinal tract (Fig. 2). Within the gastrointestinal tract, radioactivity was found both in the lumen of the small intestine (14%) and in the small intestine itself (10%), 34% was in the cecum (combined contents and mucosa), 6% was in the colon lumen, and 1% was in the colon mucosa. After the intestinal tract, the largest fractions of radioactivity were found in liver (5%) and bile (2%). Approximately 1% of the radioactivity was detected in kidneys. No radioactivity was detected in esophagus, pancreas, spleen, adrenals, brain, heart, lung, white adipose tissue, and muscle. No radioactivity was detected in systemic blood and only 1% of the dose was excreted per 100 μl of urine. Total recovery of radioactivity was 98% (±31%). In this experiment it was not further investigated whether the radioactivity in these tissues was due to cafestol or to cafestol metabolites.

Quantitative Whole-Body Autoradiography. Every mouse was cut at five different levels of its body. From every level, three sections were made. Selected whole-body autoradiograms are given in Fig. 3. QWBA confirmed the distribution pattern as obtained by dissection. Eight different time points (0–48 h) were investigated by QWBA. Thirty minutes postdose, almost all radioactivity was found in liver and intestinal tract. Trace amounts were found in systemic blood and kidneys. Apart from liver and intestinal tract, no radioactivity was seen in any other organs or tissues that were analyzed. Blood samples were collected separately, and, consistent with the data from the dissection study, no radioactivity was found. This relative distribution pattern remained essentially the same during the first 24 h postdose. At 48 h postdose, no radioactivity was detected anymore.

Biliary Excretion of Cafestol Metabolites. The biliary excretion of radioactive cafestol after intravenous administration is depicted in Fig. 4. Five hours after administration a cumulative amount of 20% of the administered dose of radioactivity was secreted in bile. In the

FIG. 2. Organ distribution of cafestol in mice. Data show the levels of [3H]cafestol (metabolites) 5 h after oral administration to mice, expressed as percentage of administrated dose corrected for organ weight. Trace amounts of cafestol were detected in total blood volume (0.001%). No cafestol could be detected in esophagus, pancreas, spleen, adrenal glands, brain, heart, lung, white adipose tissue, and muscle. Values are expressed as averages ± S.E.M. SI, small intestine.

FIG. 3. Quantitative whole-body autoradiography of cafestol-fed C57BL/6J mice. Thirty minutes postdose, almost all radioactivity is found in liver and small intestine (SI). Trace amounts were found in systemic blood and kidneys. Apart from liver and intestinal tract, no radioactivity was detected in any other sections that were analyzed. At 48 h postdose, no radioactivity was detected anymore.
blood samples taken at that moment by orbital puncture, no radioactivity was detected. HPLC analysis of bile showed that there was no parent compound detectable. Instead, several apparent cafestol metabolites were found. As can be seen in Fig. 5A, bile samples contained multiple $^3$H peaks. These peaks are designated as peak I, II, III, and IV, eluting at retention times of 3.5, 10, 13, and 18 min, respectively. To further characterize these peaks, conjugation and deconjugation reactions were performed. Deconjugation with $\beta$-glucuronidase resulted in a decrease of peak IV. A new peak (V) eluted at a retention time of 22 min, which corresponds to the parent compound cafestol (Fig. 5B). This was confirmed by conjugation reactions (data not shown) in which uridine 5-diphosphoglucuronic acid, and microsomes were added to radioactive cafestol. From the present study no direct structural information regarding the identity of the compounds can be derived. It is clear that the most abundant cafestol metabolite in bile is the cafestol glucuronide conjugate (41%). The identity of the other three metabolites, representing the majority of the radioactive cafestol metabolites in bile (retention time 3.5 min, 7%; retention time 10 min, 32%; and retention time 13 min, 20%), was not further elucidated.

Analyses of Cafestol and Metabolites in Portal Blood. Very rapidly after duodenal administration three radioactive compounds appeared in portal blood. Figure 6 shows their relative abundance in time. At all time points, the most abundant radioactive compound in portal blood was found to be the parent cafestol (50% of total radioactivity). In addition, intestinal biotransformation led to two major metabolites. One of the two has the same retention time as the glucuronide found in bile, suggesting that it is the glucuronide. At 10 min postdose 70% of parent compound is present in the portal blood. After this the concentration of parent compound decreases (to 41%), whereas at 50 min postdose, the concentration of parent compound in portal blood is increased up to 70% again. Taken together, these results indicate that absorption of parent compound and metabolites to the portal vein is prolonged and apparently follows a cyclic pattern (Fig. 6). In the sample taken from the systemic circulation at $t = 50$ min, no radioactivity was detected.

Discussion

Results from both distribution studies indicate that cafestol and/or its metabolites strongly accumulate in the liver and gastrointestinal tract. After oral administration, hardly any distribution to other parts of the body was found. This distribution pattern remains fairly constant for at least 24 h after administration. After both intravenous and oral administration no radioactive fraction corresponding to the parent compound was found in bile. This finding confirms our earlier results using liquid chromatography-mass spectrometry, with which we showed that cafestol is extensively metabolized by the liver to epoxyglutathione conjugates, glutathione conjugates, and glucuronide conjugates (van Cruchten et al., 2009). These metabolites are subsequently excreted into the bile. Although the radioactive HPLC

![Fig. 5. The metabolite at retention time 18 min is a cafestol glucuronide conjugate. A, bile sample of a mouse treated with cafestol at time point 5 min (y-axis, disintegrations per minute present in bile fraction; x-axis, retention time in minutes). B is the same bile sample but deconjugated with $\beta$-glucuronidase.](image-url)
analyses in the present study do not provide direct structural confirmation, the conjugation-deconjugation experiment confirms our previous observations that one of the major metabolites in bile is a glucuronide. The same glucuronide is also found in portal blood (already at 2 min postdose), which indicates that glucuronidation also takes place in the intestinal epithelium. However, the parent cafestol is also rapidly absorbed into the portal vein. Two minutes after dosing, the parent compound represented 50% of the total radioactivity present in portal blood. It is remarkable that cafestol absorption continued during the next 50 min, still representing 70% of the activity present in portal blood at 50 min after administration. The presence of a glucuronide in bile found to be easily deconjugated by a bacterial enzyme, together with the prolonged absorption of parent compound from the gastrointestinal tract suggests that cafestol undergoes enterohepatic cycling. It should be mentioned that the cafestol dose used in the oral studies, 1.5 mg/mouse, is rather high compared with the amount present in coffee. Depending on the brewing, coffees may contain up to 3.5 mg/cup of 100 ml (Ranheim and Halvorsen, 2005). If cafestol kinetics are nonlinear in mice, a relatively high concentration in the intestinal lumen might partly explain the prolonged absorption and accumulation in intestinal tissue. Cafestol is the most potent cholesterol-elevating compound by weight present in the human diet (Urgert and Katan, 1997). Several studies have shown that cafestol produces a clinically significant rise of serum cholesterol, which is a relevant risk for the development of cardiovascular diseases. Epidemiological studies in Scandinavians have shown that drinking large amounts of cafestol-rich coffee is indeed associated with an increased risk for coronary heart disease (Stensvold and Tverdal, 1995; Urgert et al., 1995; Urgert and Katan, 1996; Stensvold et al., 1996). High intakes have also shown to increase serum levels of alanine aminotransferase and aspartate aminotransferase, indicating liver damage (Urgert and Katan, 1997; Urgert et al., 1997). In a previous study we proposed that epoxidation of the furan ring plays a role in these hepatotoxic effects of cafestol (van Cruchten et al., 2009). We also showed in vitro induction by cafestol of an EpRE in human HepG2 cells (van Cruchten et al., 2009). We also showed in vitro induction by cafestol of an EpRE derived from the human NQO1 regulatory region (van Cruchten et al., 2009). Further studies are needed to elucidate in which form accumulation, and cholesterol elevation remain to be elucidated. The effect of cafestol on blood lipids in humans is unusually slow. It takes at least 4 weeks to reach new steady-state levels of blood lipids. Our data do not suggest that this is due to a slow accumulation of cafestol in some body pool, because cafestol does not seem to penetrate beyond the enterohepatic axis and is cleared fairly rapidly. Secondary changes in liver metabolism induced by cafestol may explain its unusually protracted effect on blood lipids.

In conclusion, data from the present study provide evidence that cafestol is efficiently absorbed and partially metabolized by the gut, cleared and further metabolized by the liver, and excreted into bile. Because the main metabolite is a glucuronide and because the parent compound appears to be present in portal blood, we suggest that cafestol is likely to undergo enterohepatic circulation.

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References
Cavin C, Holthausen D, Scharf G, Constable A, Huber WW, and Schilter B (2002) Cafestol and the natural toxin ipomeanol, formation of furan epoxides has indeed been associated with cellular toxicity. To the best of our knowledge, no evidence for the presence of either cafestol or its metabolites in human plasma is available. In the present study, we also found no cafestol in the peripheral circulation of mice that received cafestol orally. The only human data on cafestol pharmacokinetics and metabolism formation in more detail have been published by De Roos et al. (1998). These colleagues investigated cafestol disposition in healthy ileostomy volunteers. From the recovery of cafestol metabolites in the ileostomy effluent, it was estimated that approximately 70% was absorbed from the gastrointestinal tract. As only approximately 1% of the dose was recovered in urine, it was concluded from that study that cafestol is subject to extensive metabolism in the human body. These observations in humans are in line with those of the present study, but our new data put the original conclusion in a slightly different perspective. Indeed, cafestol seems to be absorbed very rapidly and its passage to the systemic circulation is near zero. However, in addition to direct biotransformation, accumulation in the liver and enterohepatic cycling play important roles in the kinetics of the compound. The causal relations between epoxide formation, liver accumulation, and cholesterol elevation remain to be elucidated. The effect of cafestol on blood lipids in humans is unusually slow. It takes at least 4 weeks to reach new steady-state levels of blood lipids. Our data do not suggest that this is due to a slow accumulation of cafestol in some body pool, because cafestol does not seem to penetrate beyond the enterohepatic axis and is cleared fairly rapidly. Secondary changes in liver metabolism induced by cafestol may explain its unusually protracted effect on blood lipids.

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