**ABSTRACT:**

The metabolism of apigenin, a low estrogenic flavonoid phytochemical, was investigated in rat using liver models both in vitro (subcellular fractions) and ex vivo (isolated perfused liver). In vitro, phase I metabolism led to the formation of three monohydroxylated derivatives: luteolin which was the major metabolite ($K_m = 22.5 \pm 1.5 \mu M; V_{max} = 5.605 \pm 0.090 \text{ nmol/min/mg protein}, \text{ means} \pm \text{S.E.M.}$), scutellarenin, and iso-scutellarenin. These oxidative pathways were mediated by cytochrome P450 monoxygenases (P450s). The use of P450 inhibitors and inducers showed that CYP1A1, CYP2B, and CYP2E1 are involved. In vitro studies of phase II metabolism indicated that apigenin underwent conjugation giving three monoglucuronon conjugates and one monosulfon conjugate. Luteolin led to the formation of four monoglucuronon conjugates, two sulfoconjugates, and one methylconjugate identified as diosmetin. Ex vivo during the apigenin perfusion of an isolated rat liver, none of the phase I metabolites could be recovered. In contrast, two monoglucuronon conjugates and one of the sulfoconjugates of apigenin already identified in vitro were recovered. Moreover, two new derivatives were isolated and identified as a diglucurononoconjugate and a glucuronosulfon conjugate. This work provides new data about the metabolism of apigenin and shows the interest value of using various experimental models in metabolic studies.

Flavonoids are naturally occurring polyphenolic compounds that are ubiquitous in plants. The average daily human intake of flavonoids in North European countries has been estimated at up to 20 mg (Havsteen, 2002). They have a variety of biological effects in numerous mammalian systems in vitro as well as in vivo: free radical scavengers, antioxidants, pro- or antimutagens, and anti-inflammatory, antiviral, or purgative effects. Some of them have been noted for their beneficial effect on cardiovascular diseases and cancer prevention (Suschetet et al., 1998; Middleton et al., 2000). These effects could be explained by their abilities to inhibit the cell cycle, cell proliferation, or oxidative stress, improve the efficacy of detoxification enzymes, induce apoptosis, and stimulate the immune system (Suschetet et al., 1998; Birt et al., 2001; Havsteen, 2002). Recently, much attention has been paid to their endocrine properties and their potential roles on fertility and hormone-dependent diseases. Some of the effects of soy isoflavones (e.g., genistein, daidzein) such as alterations of the reproductive tract in mammalians (Middleton et al., 2000), prevention of osteoporosis and menopausal symptoms (Havsteen, 2002), or antitumorigenic effects (Birt et al., 2001), may be related to estrogen-related mechanisms (Middleton et al., 2000; Cotroneo et al., 2001). Some 5,7,4’-hydroxyflavonoids (Fig. 1) present in the Western diet such as apigenin, luteolin, kaempferol, or naringenin have also been shown to possess estrogenic or antiestrogenic properties in vitro (Breinholt and Larsen, 1998; Le Bail et al., 1998; Middleton et al., 2000; Birt et al., 2001) as well as in vivo (Breinholt et al., 2000; Hiremath et al., 2000). They are about 1000 times less estrogenic than genistein (Middleton et al., 2000), but their presence in great amounts could explain the abortive properties of some medicinal plants (Hiremath et al., 2000).

Apigenin has the same hydroxylamination pattern as genistein. It is found in apple, celery, tea, aromatic plants, and honey (Havsteen, 2002). Its anticancer properties have been extensively studied in vitro and in vivo (Birt et al., 2001; Havsteen, 2002), as it can enhance gap- junction intercellular communication and induce apoptosis (Suschetet et al., 1998; Wang et al., 1999). It modulates the activities of cytochrome P450 monoxygenase (P450) and an isoforom involved in bioactivation of numerous carcinogens (Allen et al., 2001), and it is a ligand of estrogen receptor (Breinholt and Larsen, 1998; Havsteen, 2002). Apigenin was shown to strongly inhibit the proliferation of keratinocytes (Lepley et al., 1996) and prostate cancer cells (Gupta et al., 2001). Indeed, it was described as a preventive agent in mouse skin tumorigenesis (Birt et al., 2001).

Even though knowledge on the potential benefits of apigenin on...
Health is increasing, few data about its absorption, distribution, metabolism, and excretion in human or in experimental models such as rodents have been published. These data are essential to identify active compounds and to explain their mechanisms of action. Mammalian biotransformations of isoflavonoids and flavonoids could modulate their biological properties, since sulfate and glucuronide derivatives are generally less effective than the aglycone forms (Birt et al., 2001). Sulfate and glucuronide derivatives of apigenin have been recovered in human urine (Nielsen and Dragsted, 1998) or in human carcinogenic cell cultures (Galijatovic et al., 1999). Such conjugates have been also identified in rat urine (Havsteen, 2002). However, in vitro studies show that apigenin is oxidized into luteolin by rat liver microsomes (Nielsen and Dragsted, 1998). This phase I metabolite could be methylated by rats in vivo (Liu et al., 1995) and glucuronidated by rat liver microsomes (Boutin et al., 1993). Nevertheless, even though the importance of apigenin in vivo metabolism on its estrogenic activity was suggested (Breinholt et al., 2000), data on its metabolism, and excretion in human or in experimental models such as rodents have been published. These data are essential to identify active compounds and to explain their mechanisms of action. Mammalian biotransformations of isoflavonoids and flavonoids could modulate their biological properties, since sulfate and glucuronide derivatives are generally less effective than the aglycone forms (Birt et al., 2001). Sulfate and glucuronide derivatives of apigenin have been recovered in human urine (Nielsen and Dragsted, 1998) or in human carcinogenic cell cultures (Galijatovic et al., 1999). Such conjugates have been also identified in rat urine (Havsteen, 2002). However, in vitro studies show that apigenin is oxidized into luteolin by rat liver microsomes (Nielsen and Dragsted, 1998). This phase I metabolite could be methylated by rats in vivo (Liu et al., 1995) and glucuronidated by rat liver microsomes (Boutin et al., 1993). Nevertheless, even though the importance of apigenin in vivo metabolism on its estrogenic activity was suggested (Breinholt et al., 2000), data on its behavior in experimental models are lacking.

The aim of this work was to acquire more information concerning hepatic metabolism of apigenin in rats, which are the usual model in studies concerning carcinogenesis, endocrine screening tests, or in vitro mechanism studies. At first, phase I and phase II metabolism of apigenin was investigated in vitro using liver subcellular fractions to assess metabolic formation and to identify the enzymes involved. Second, an isolated perfused liver system provided information on the pharmacokinetic parameters of apigenin. In this ex vivo model, all of the metabolizing enzyme are present and may affect competing reactions. This work enabled us to identify new metabolites of apigenin and to increase our knowledge about flavonoid metabolism in rat liver.

Materials and Methods

Chemicals. Apigenin, luteolin, diosmetin, iso-scutellarein, and scutellarein were purchased from Extrasynthese (Genay, France; purity >90%). 1-Aminobenzotriazole, α-naphthoflavone, cimetidine, diethylthiokarbamate, methimazole, metyrapone, orphenadrine, quinidine, sulfaphenazole, S-adenosyl-L-methionine, troleandomycin, uridine diphosphoglucuronic acid, sulfatase (type VI), and sulfoglucuronyltransferase (type H-3AF) were purchased from Sigma-Aldrich Chimie (St.-Quentin Fallavier, France). Arochlor 1254 was purchased from AccuStandard, Inc. (New Haven, CT). All other chemicals were purchased from standard commercial sources and were of the highest quality available.

Animals and Treatments. Weaned male and female SPF Wistar rats were obtained from Elevage Janvier (Le Genest Saint Isle, France). They were housed individually in wire cages in a room maintained at 22°C with a 12-h light-dark period. Males were fed ad libitum for 4 weeks on a purified diet for in vitro experiments. P450 inducers (acetone, clofibrate, dexamethasone, methylcholanthrene, phenobarbital) were administered at the end of this period as previously described by Teyssier and Siess (2000). The arochlor 1254 induction was performed by a single intraperitoneal injection of a solution of arochlor dissolved in corn oil at the dose of 500 mg/kg body weight 5 days before sacrifice. Female and male rats for ex vivo experiment were fed ad libitum for 1 week on a semisynthetic, phytoestrogen-free diet.

Preparation of Subcellular Fractions. Male rats were killed by cervical dislocation and exsanguination after 16 h of fasting. Hepatic subcellular fractions and quantification of microsomal or cytosolic proteins and of P450 were performed as previously described (Teyssier and Siess, 2000).

Phase I Enzyme Assay. For the oxidation reaction, a mixture containing 60 to 80 mM KCl-Tris buffer (pH 7.4), 0.5 mg/ml microsomal protein, and 1 mM NADPH in a 0.5-ml final volume was preincubated at 37°C for 3 min. The reaction was started by adding 0.5 mM flavonoid (dissolved in dimethyl sulfoxide). The reaction was performed at 37°C and stopped after 10 min by the addition of 50 μl of trichloroacetic acid (30%). After centrifugation (10 min, 2000g), the supernatant was analyzed by HPLC.

Thermal inactivation of flavin-containing monoxygenases (FMOs) in microsomes was performed by precubation of the above mixture but without NADPH for 10 min at 37°C. In parallel, controls were preincubated in the presence of NADPH (which protects the enzymes from denaturation). Then apigenin and NAPDH were added to both samples before the 10-min incubation. Methimazole oxidase activity was checked to confirm inactivation of FMO at 37°C as previously described (Teyssier and Siess, 2000). P450 inhibitors were added to the incubation mixture, which contained noninduced microsomes to assess the enzymes involved (Teyssier and Siess, 2000). The inhibitors were dissolved in ethanol and the concentration of ethanol in the incubation medium was less than 0.1%. As 1-aminobenzotriazole, diethylthiothiocarbamate, and troleandomycin are mechanism-based inhibitors, they were preincubated for 10 min before starting the reaction.

Kinetic studies were performed in the same conditions as described for the oxidation reaction with apigenin concentrations of 0, 0.02, 0.04, 0.08, 0.16, 0.28, and 0.50 μM, and Km and Vmax values were estimated by substitution into the Michaelis-Menten equation using a nonlinear regression program of the SAS software (SAS Institute, Inc., Cary, NC).

Phase II Enzyme Assay. Glucuronon conjugation was investigated by incubating microsomal proteins (0.5 mg/ml) at 37°C for 2 h in a medium (0.5 ml) containing 80 mM Tris buffer (pH 7.4), 5 mM MgCl2, 2 mM uridine diphosphoglucuronic acid, and 0.5 mM flavonoid. The reaction was stopped by the addition of 1 N HCl. For the oxidation reaction, a mixture containing 60 to 80 mM KCl-Tris buffer (pH 7.4), 0.5 mg/ml microsomal protein, and 1 mM NADPH in a 0.5-ml final volume was preincubated at 37°C for 3 min. The reaction was started by adding 0.5 mM flavonoid (dissolved in dimethyl sulfoxide). The reaction was performed at 37°C and stopped after 10 min by the addition of 50 μl of trichloroacetic acid (30%). After centrifugation (10 min, 2000g), the supernatant was analyzed by HPLC.

Sulfation was investigated by incubating cystolic proteins (1 mg/ml) at 37°C for 2 h in a medium (0.5 ml) containing 40 mM Tris buffer (pH 7.4), 5 mM MgCl2, 3 mM mercaptoethanol, 0.2 mM 3-phosphoadenosine-5′-phosphosulfate, and 0.5 mM substrate. The reaction was stopped by adding 500 μl of cold methanol. After centrifugation (10 min, 2000g), the supernatant was analyzed by HPLC and LC-MS. Sulfatase assay was performed as follows: 0.2 units of sulfatase were added to the samples which were then incubated for 2 h at 37°C.

The mixed reaction of conjugation (sulfot- and glucuronon conjugation) was investigated by incubating microsomal and cystolic proteins (0.5 and 1 mg/ml, respectively) at 37°C for 3 h in a medium (0.5 ml) containing 50 mM Tris buffer (pH 7.4), 5 mM MgCl2, 3 mM mercaptoethanol, 2 mM uridine diphosphoglucuronic acid, 0.2 mM phosphoadenosine-5′-phosphosulfate.
(PAPS), and 1 mM substrate. The reaction was stopped by adding 500 µl of cold methanol. After centrifugation the supernatant was analyzed by HPLC and LC-MS.

The reaction of methylation was investigated by incubating cytosolic proteins (1 mg/ml) at 37°C for 2 h in a medium (0.5 ml) containing 50 mM Tris buffer (pH 7.4), 1.2 mM MgCl₂, 200 µM S-adenosyl-methionine, 1 mM dithiothreitol, and 0.5 mM substrate. The reaction was stopped by adding 1 N HCl. After centrifugation the supernatant was analyzed by HPLC.

**Liver Perfusion.** Livers (n = 5 of each sex) were isolated as previously described (Teyssier and Siess, 2000). Briefly, the perfusion medium consisted of Krebs-Henseleit bicarbonate buffer, pH 7.4, containing glucose (0.1 mM final concentration), heated to 37°C and saturated with an oxygen-carbon dioxide mixture (95:5). The perfusion of the liver was realized via the cannulation of the portal vein at a constant flow rate (15 ml/min). After a single-pass equilibration period, the experiments were performed with recirculation of a new solution of perfusate containing 100 µM apigenin (reservoir of 150 ml). Samples (0.8 ml) were collected at 0, 5, 10, 15, 20, 30, 45, 60, and 75 min following initiation of the apigenin perfusion. The viability of the organ was checked by measuring the lactate dehydrogenase activity for each time-sample by a direct spectrophotometric method as previously described (Teyssier and Siess, 2000).

Time-samples from male rats were analyzed only by direct injection in HPLC. Time-samples from female rats were analyzed directly and after the action of glucuronidase or sulfatase enzymes (as previously described) to check the nature of the metabolites obtained. At the end of the experiment, the final perfusion medium was acidified and frozen (−80°C) until LC-MS analysis.

The pharmacokinetic parameters were calculated with Kinetica software (InnaPhase, Champs sur Marne, France) using the noncompartmental method. The elimination rate constant (kₑ) was determined by linear regression of the log perfusate concentrations in the elimination phase ß. The area under the curve (AUC) was calculated from the last point to infinity with kₑ. The half-life (t₁/₂) was determined from the β phase in the log-linear regression plot. Clearance was deduced from the dose/area under the curve ratio and the distribution volume from the clearance/kₑ ratio.

**HPLC Analysis.** The HPLC system consisted of a Waters (Saint Quentin-Yveline, France) system with a pump (model 600), an autosampler (model 717), and a photodiode array UV detector (model 996).

Hydroxylated and methyl conjugates of apigenin and liver perfusate samples were separated in an Uptisphere 5 µm ODB column (4.6 × 150 mm; Interchim, Montluçon, France). The mobile phase consisted of a mixture of solvent A (0.1% diethylamine buffer, pH 3.0) and solvent B (acetonitrile) and was delivered at a constant flow rate of 2 ml/min. The gradient started at 70% A, 30% B for 5 min, followed by a linear increase of solvent B to 70% in 3 min. The initial conditions were returned to in 2.5 min and followed by a stable phase of 2.5 min.

The mobile phase for the separation of sulfo- and glucuronon conjugates consisted of a mixture of solvent C (0.5% formic acid solution) and solvent D (acetonitrile containing 0.5% formic acid) and was delivered at a constant rate of 1 ml/min. The gradient started at 90% C, 10% D for 5 min, followed by a linear increase of solvent D to 30% from min 5 to min 25. This rate composition was maintained from min 25 to min 40, and initial conditions were returned to in a linear mode from min 40 to min 45. The column was then equilibrated for 15 min.

The spectra were obtained with the photodiode array detector from 200 to 400 nm. The quantities of apigenin and metabolites were measured at 340 nm. The quantities of derivatives were performed when standards were available on the basis of calibration curves plotted with duplicate injections (minimum of four concentrations). The lowest level of quantification was about 18 pmol, and the coefficient of variation was 20%. The quantification of apigenin during the perfused liver experiment was performed using a standard curve of apigenin spiked in the perfusion medium. The limit of detection was about 16 pmol.

**LC-MS Analysis.** Analyses of the metabolites were performed with the HPLC conditions described above. An interface was established between a Waters system (Milford, MA), including pump (model 2690), autosampler, and photodiode array detector (model 996), and a single quadrupole Waters LCZ Platform (Waters) equipped with an electrospray ionization source operating in negative ionization mode. Mass spectra were acquired in selective ion-recording mode or full scans from m/z = 200 to 700 at 1 scan/s. The following conditions were used: electrospray ionization capillary voltage, 3.50 kV; temperature desolvation, 150°C; desolvation gas, 400 l/h; and fragmentation voltage, 20 V.

**Statistical Analysis.** Data from the inactivation of FMO experiment were subjected to a Student’s t test (p ≤ 0.05%). Data from the induction and inhibition experiments were subjected to analysis of variance followed by Dunnett’s test (p ≤ 0.05) using the SAS system.

**Results**

**Metabolism by Phase I Enzymes.** After incubation of a medium containing the arachlor-induced microsomes, apigenin, and NADPH, three peaks were detected: two minor peaks corresponding to HA1 and HA2, and one major peak corresponding to metabolite HA3. They eluted at 2.47, 2.79, and 3.59 min, respectively. When an incubation medium containing noninduced rat microsomes was used, only HA3 was detected. The formation of these metabolites was dependent on the presence of NADPH (data not shown). It appeared to be linear with respect to incubation time (up to 20 min) and microsomal protein

![Fig. 2. Luteolin formation by incubation of control rat liver microsomes (A) or arachlor-induced rat liver microsomes (B) with 500 µM apigenin. Values are means ± standard error mean (n = 4).](image-url)
concentration (up to 1 mg/ml). Metabolite HA3 formation followed monophasic Michaelis-Menten kinetics, and the estimated $K_m$ and $V_{max}$ values were 10.37 ± 1.56 μM and 0.0108 ± 0.003 nmol/min/mg protein, respectively (Fig. 2A). When the reaction was performed with arochlor-induced microsomes, $V_{max}/K_m$ was 10 to 30 times higher for HA3 (329.7) compared with HA1 and HA2 (30.1 and 11.7, respectively), suggesting that the formation of HA3 was the major metabolic pathway of apigenin.

$K_m$ values were 13.9 ± 1.7, 31.9 ± 2.9, and 22.5 ± 1.5 μM; and $V_{max}$ values were 0.421 ± 0.028, 0.376 ± 0.042, and 5.605 ± 0.090 nmol/min/mg protein for HA1, HA2, and HA3, respectively (Fig. 2B).

LC-MS analysis of HA1, HA2, and HA3 showed deprotonated ions ([M-H]$^-$) with an $m/z$ of 285, which correspond to monohydroxylation of apigenin. Flavones have a characteristic fission in the C ring (Hedin and Phillips, 1992; Havsteen, 2002), which was observed in our conditions (Fig. 3A). The position of an additional group can be deduced by the mass of each fragment obtained after fission (Fig. 3B). Fragment ions were observed at $m/z$ 167.1 after MS/MS fragmentation of HA1 and HA2, whereas an $m/z$ of 151 was obtained for HA3. This indicated that HA1 and HA2 possess an additional hydroxyl group on A ring, whereas HA3 has one on B ring. There are two possibilities of hydroxylation in the A ring: C6 (corresponding to scutellarein, 4',5,6,7 tetrahydroxy flavone) and C8 (corresponding to iso-scutellarein, 4',5,7,8 tetrahydroxy flavone). From the HPLC retention time and the spectrum profile of these compounds compared with standards, HA1, HA2, and HA3 were identified as scutellarein, iso-scutellarein, and luteolin, respectively (Fig. 1).

As the metabolism of apigenin occurred only in the presence of NADPH, P450 or FMO is mainly involved. Following thermal inactivation of FMO, apigenin metabolism did not decrease (97.4 ± 10.5% of the control in the absence of FMO). When the reaction took place in the presence of 1-aminobenzotriazole, which is a suicidal-aspecific P450 inhibitor, metabolite formation is reduced by 87% (Fig. 4). These results suggest that P450s are the main mediators in phase I enzyme reactions. To determine which isoform of P450 could be involved in the oxidative metabolism of apigenin, two complementary approaches using specific P450 inhibitors and inducers were assessed.

When apigenin was incubated with noninduced rat microsomes, only luteolin formation was recovered and measured. Using P450-induced rat microsomes (Fig. 5), luteolin formation was increased (39% of induction) by arochlor (CYP1A and -2B) but was not modified by the use of MC (CYP1A), phenobarbital (CYP2B/3A), acetone (CYP2E1), dexamethasone (CYP3A), or clofibrate (CYP4A). The minor metabolites scutellarein and iso-scutellarein were only recovered in arochlor- and MC-induced rat liver microsome incubations.

When apigenin was incubated with control rat liver microsomes in the presence of the specific P450 inhibitors α-naphthoflavone (CYP1A), quinidine (CYP2D1), cimetidine (CYP2C11), or sulfafenazole (CYP2C6), no effect was observed (Fig. 4). Surprisingly, the formation of luteolin was strongly inhibited by diethylthiocarbamate (100 μM, 79.3%) and to a lesser extent by orphenadrine (200 μM, 61%)}
Values are means ± standard error mean (n = 4), and asterisk indicates significant difference from control (Co) (Dunnett’s test p < 0.05). Ar, arachidol; AC, acetone; Cl, clofibrate; DX, dexamethasone; PB, phenobarbital.

34.4%), suggesting the involvement of CYP2E1/3A and CYP2B/2C, respectively, in the control liver microsomes. Taken together, the use of P450 inducers and inhibitors indicates that several P450 isoforms are involved in apigenin oxidative metabolism.

**Metabolism by Phase II Enzymes. Glucuronidation reaction.** When apigenin was used as the substrate for the glucuronidation reaction, three peaks were detected by HPLC, namely MGA1 (major peak) and MGA2 and MGA3 (minor peaks), all three of which disappeared after β-glucuronidase hydrolysis. LC-MS analysis indicated a molecular ion at m/z 445, suggesting monoglucuronides. When luteolin was used as the substrate, four peaks were detected by HPLC. These disappeared after incubation with β-glucuronidase. LC-MS analysis suggested monoglucuronides with an m/z of 461. They were namely MGL1, MGL2, MGL3, and MGL4 (Table 1).

LC-MS analysis of apigenin conjugates and mass fragmentation did not result in typical flavonoid fragmentation. The glucuronoyl ether link seemed to be unstable, and determination of the substituted cycle was impossible.

**Sulfation reaction.** When apigenin was used as the substrate for the sulfation reaction, one peak was detected by HPLC, namely MSA1 (Table 1), whereas luteolin led to the formation of two metabolites: MSL1 and MSL2. All these derivatives disappeared after sulfatase hydrolysis. LC-MS analysis revealed an m/z of 349 for the apigenin metabolite and an m/z of 365 for the luteolin metabolites, indicating monosulfoconjugation (Table 1).

**Mixed-conjugation reaction (sulfate and glucuronyl group).** When apigenin and luteolin were submitted to the double reaction of conjugation (sulfo- and glucurononoconjugation), the HPLC profile was similar to the elution profile of the glucuronidation medium. No mixed conjugates were observed in our experimental conditions.

**Methylation reaction.** Methylation assays were also performed with apigenin and luteolin. No product was formed when apigenin was used as the substrate. The use of luteolin led to the formation of one metabolite, MLI, which was identified as diosmetin (4'-methyl luteolin) by comparison with HPLC retention time and absorption spectrum of the standard (Table 1 and Fig. 1).

**Metabolism in a perfused liver.** In this experiment, the release of lactate dehydrogenase was used to evaluate the viability of the livers. Lactate dehydrogenase activity slowly increased during the perfusion period and did not exceed 431.33 ± 163.91 U/l (mean ± S.E.M.) at 75 min. The sizes of the livers were 111.1 ± 84.4 g with a flow rate of 1.38 ± 0.10 ml/min/g (means ± S.E.M.). In a recirculating system, apigenin rapidly disappeared from the perfusion medium. Figure 6 shows the linear representation of mean concentrations versus time. Apigenin exhibited a biphasic decline. A rapid distribution phase was followed by a second slowest elimination phase. The calculated pharmacokinetic parameters of both experiments are summarized in Table 2. Time-sample HPLC analysis revealed a time-related increase in the formation of compounds that eluted before apigenin. When the female samples were subjected to enzymatic digestion by β-glucuronidase, these compounds disappeared and were replaced by apigenin. LC-MS analysis of the perfusate of female rats at the end of the experiment revealed the presence of monoglucurononoconjugates (m/z 445), monosulfoconjugates (m/z 349), and monoglucurononoconjugate (m/z 525) formed in a double-reaction (glucuronono- and sulfoconjugation) (Fig. 7). The monoglucurononoconjugates corresponded to MGA1 and MGA2 (apigenin metabolites) observed during in vitro assay. The monosulfoconjugate corresponded to MSA1. The derivative formed in a double reaction corresponded to a monosulfoconjugate derivative of apigenin namely MGMSA1. A diglucurononoconjugate of apigenin was also detected in 80% of the analyzed samples, with a mass m/z of 621, showing that dissubstitution is possible.

**Discussion**

The use of in vitro and ex vivo experimental models provides new data concerning apigenin metabolism in rat liver. According to previous work, apigenin is converted in vitro into a major metabolite, luteolin (Liu et al., 1995; Nielsen et al., 1998). Two novel hydroxylated metabolites have been revealed and identified, namely scutellarin and iso-scutellarin and iso-scutellarin (Fig. 8). Both inactivation of FMO and inhibition of P450s show that P450 family enzymes were involved in these oxidations. Since the isoenzyme-specific inhibitors α-naphthoflavone, quinidine, sulfaphenazole, cimetidine, and troleandomycin did not interfere with luteolin formation, the corresponding P450 isoforms did not seem to be involved in phase I apigenin metabolism. In contrast, the decrease observed in the presence of the inhibitors orphenadrine and diethyldithiocarbamate (65 and 30% of control) indicated that CYP2B and CYP2E1 were involved. Surprisingly, when apigenin metabolism was studied using microsomes from male rats treated with the corresponding inducing agent, neither phenobarbital (CYP2B) nor acetone (CYP2E1) inducers increased luteolin formation. In the same manner, as the use of dexamethasone- and clofibrate-induced microsomes had no effect on luteolin formation, CYP3A and CYP4A did not seem to be involved. In contrast, MC did not produce changes in luteolin formation; however, only this specific P450 inducer allowed scutellarin and iso-scutellarin production, suggesting that CYP1A1 involvement was minor in phase I apigenin.
metabolism. These results differ from those found in previous works which showed that CYP1A2 and CYP3A4 were involved in apigenin metabolism mediated by human and mouse subcellular fractions (Breinholt et al., 2002). Nevertheless, the involvement of CYP1A isoform has already been described in the metabolism of several flavonoids such as kaempferol, tamarixetin (Silva et al., 1997; Nielsen et al., 1998), and tangeretin (Canivenc-Lavier et al., 1993; Nielsen et al., 2000). All these data suggest the implication of several P450 isoforms, sustaining the idea that apigenin or flavonoid metabolism results from a multienzyme involvement.

Glucuronidation, sulfation, or methylation, which are conjugation reactions usually observed in flavonoid metabolism (Havsteen, 2002), was also observed in our study. No methyl derivative of apigenin was found in our conditions, which agrees with the results of Nielsen et al. (1999). In contrast, we observed the formation of one methylated luteolin metabolite, thus confirming the existence of a derivative compound already detected in vivo (Liu et al., 1995; Shimoi et al., 1998). This compound was identified as 4'-methyl luteolin, i.e., diosmetin (Fig. 8).

Despite many available conjugation sites on flavonoid molecules, diglucuronides were not observed under our conditions. All the glucuronocojugates of apigenin (MGA1, MGA2, MGA3) and luteolin (MGL1, MGL2, MGL3, MGL4) were monosubstituted (Fig. 8). This finding is in accordance with previous results concerning monosubstituted derivatives formed in vitro with quercetin (Boersma et al., 2002), diosmetin (Boutin et al., 1993), or luteolin (Shimoi et al., 1998). The diglucuronocojugates described in the literature were observed in vivo (Abe et al., 1990; Boutin et al., 1993).

Differences in metabolite formation confirmed the existence of preferential conjugation sites, such as C7 and C3', as has already been suggested by Havsteen (2002). These specific sites may be related to the steric configuration of flavones. When glucuronocojugates of apigenin or luteolin have been described (Griffiths, 1964; Boutin et al., 1993; Galijatovic et al., 1999), no indication of the number or the location of the substitution sites has been given. Under our conditions, we detected three derivatives of apigenin, suggesting that at least three different substitution sites are involved. Monoglucuronides of luteolin have already been described in vivo in rat urine (Boutin et al., 1993; Shimoi et al., 1998), and in rat jejunum perfusion, C7 conjugation was predominant (Spencer et al., 1999). Recently, NMR analysis of luteolin incubated with rat microsomes resulted in the production of three monoglucuronides, probably substituted on C7, C4', and C3' with predominance of conjugation on C7 and C4' (Boersma et al., 2002).

Our study indicates four glucuronoderivatives of luteolin substituted on C7, C4', C3', and C5, which correspond to an additional metabolite. Data concerning the number of apigenin and luteolin derivatives indicate that every free site of the flavonoid molecule can be substituted (either C5, C7, C3', or C4').

Isoforms of UGTs implicated in glucuronidation were not tested in this study but the literature provides many examples. Oliveira and Watson (2000) suggested the involvement of UGT1A9, whereas Galijatovic et al. (1999) showed the involvement of UGT1A6. Apigenin was also described as a good substrate for the UGT 1.1 isoform (King et al., 1996).

Concerning sulfation reactions, one and two monosulfoconjugates of apigenin and luteolin, respectively, were observed (Fig. 8). To our knowledge, this is the first time that sulfoapigenin derivatives were obtained using subcellular fractions. This result can be related to

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male Rat</th>
<th>Female Rat</th>
</tr>
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<tbody>
<tr>
<td>Half-life (min)</td>
<td>12.04 ± 3.97</td>
<td>18.79 ± 2.23</td>
</tr>
<tr>
<td>Clearance (ml/min)</td>
<td>13.0 ± 0.0</td>
<td>13.7 ± 3.0</td>
</tr>
<tr>
<td>Area under the curve (min · nmol/ml)</td>
<td>1273 ± 150</td>
<td>1289 ± 241</td>
</tr>
<tr>
<td>Volume of distribution (ml)</td>
<td>212 ± 70</td>
<td>354 ± 63</td>
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</tbody>
</table>

Results are means of five replicate experiments ± S.E.M.
previous in vitro studies showing that flavonoids (apigenin, kaemperol, and quercetin) compete with resveratrol for sulfation reactions (De Santi et al., 2000). In the same way, cell culture studies showed that SULT1A1 and SULT1A3 enzymes were involved in chrysirin metabolism in rats (Galijatovic et al., 1999). Similar results concerning epicatechin sulfation by liver cytosols were also found in humans (Havsteen, 2002).

Concerning mixed conjugations (with glucuronic acid and sulfate substitution), data from the literature show contradicting results: in vitro and in vivo studies did not reveal mixed derivatives, whereas ex vivo experiments did (Galijatovic et al., 1999; Havsteen, 2002). In our in vitro experimental conditions, no mixed conjugate of apigenin or luteolin was obtained.

Ex vivo studies using an isolated perfused liver produced one mixed conjugate (MGMSA1) in accordance with an earlier study on perfused rat liver systems (Shali et al., 1991). The only metabolites obtained during perfusion were conjugates of apigenin; no luteolin derivative was found, suggesting that the oxidation of apigenin observed in vitro was not the main elimination route in this ex vivo model (Fig. 8). This difference can be explained by the fact that in a study of perfused rat liver, enzymes are a system of close competition. The formation of metabolites is thus closer to what happens in vivo.

In this agreement with the in vivo results observed in humans (Nielson and Dragsted, 1998) or in rats with other flavonoids such as daidzein, genistin, baicalin, and quercetin (Yasuda et al., 1994; Sfakianos et al., 1997; Middleton et al., 2000). In our competitive enzyme system, glucurono-, sulfo-, and mixed glucuronosulfon conjugates were obtained (Fig. 8). Both glucuronidation and sulfation seem to be important, and it would be very interesting to quantify the compounds produced in organisms. According to Kasper and Henton (1980), mainly sulfate products were recovered in mammal metabolism, and in cell culture the production of sulfate compounds was twice as high as that of glucuronyl compounds. Nevertheless, sulfotransferases can be rapidly saturated, leading to a greater production of glucuronyl compounds (Koster et al., 1981; Huang et al., 1997), metabolism could thus depend on the doses of apigenin effectively ingested.

Like its main hydroxylated metabolite, luteolin, apigenin is an estrogenomimetic compound. In vitro both compounds can bind to estrogen receptors (Middleton et al., 2000), and in vivo both induce uterus weight increase and antifertility (Hiremath et al., 2000). The mode of action of apigenin is still unclear, but metabolism is the first step that can help to build our knowledge. Moreover, as estrogens, apigenin, and other 5,7,4’-trihydroxylated flavonoids interact with P450 and phase II enzymes involved in the metabolism of estrogens, there may be competition between flavonoids and endogenous substrates such as steroids, which could lead to an alteration of sex hormone levels (Havsteen, 2002).

This is an important step in the comprehension of the metabolism of apigenin in a complete study with rat subcellular fractions. It will be completed by in vivo experiments to determine pharmacokinetic parameters and metabolite formation in animals and to evaluate the impact of daily absorption on health.

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References


