Implication of Steroid Saponins and Sapogenins in the Hypocholesterolemic Effect of Fenugreek

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The transformation of fenugreek subfractions, rich in steroid saponins, was studied upon their passage through the digestive tract to determine the contribution of saponins and/or diosgenin and other steroid sapogenins to the hypocholesterolemic effect of fenugreek seeds. Feces of alloxan diabetic dogs fed fenugreek subfractions were analyzed, and diosgenin, smilagenin and gitogenin were identified and measured using capillary gas chromatography/mass spectrometry. Our results show that saponins are, in part (about 57%), hydrolyzed into sapogenins in the digestive tract. It appears that saponins may be implicated, alone or together with diosgenin, in the observed hypocholesterolemic effect of fenugreek seeds in diabetic dogs.

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Fenugreek (*Trigonella foenum graecum L., Leguminosae*) is an annual herb, cultivated in Southern Europe, Northern Africa and India. The seeds of fenugreek are known to have hypocholesterolemic activity (1,2). We have previously shown that this hypocholesterolemic effect was due to the defatted part (3) and especially to the saponin-rich subfraction (4). This subfraction, which contains most of the saponins present in the seed, reduced hypercholesterolemia and hypertriglyceridemia of alloxan-diabetic dogs. The hypocholesterolemic effect was not observed with the saponin-free subfraction (4).

Saponins are plant glycosides whose aglycone structure is triterpenoid or steroidal. Studies on the effects of saponins on cholesterol homeostasis concern mainly the triterpenoid saponin from alfalfa (5) and from soybean (6). It has also been reported that a steroidal saponin, digitonin, prevents hypercholesterolemia in monkeys (7).

Fenugreek seed saponins are of steroidal nature (type furostanol saponins) with diosgenin [(25 R)-spirost-5-en-3β-ol] as main sapogenin (Fig. 1) (8). Diosgenin has various effects on cholesterol metabolism, one of the most important being the capacity to lower plasma cholesterol concentration (9). This hypocholesterolemic effect appears to be dependent on the capacity of diosgenin to inhibit cholesterol absorption, to decrease liver cholesterol concentration and to increase biliary cholesterol secretion and fecal excretion of neutral sterols (10–12).

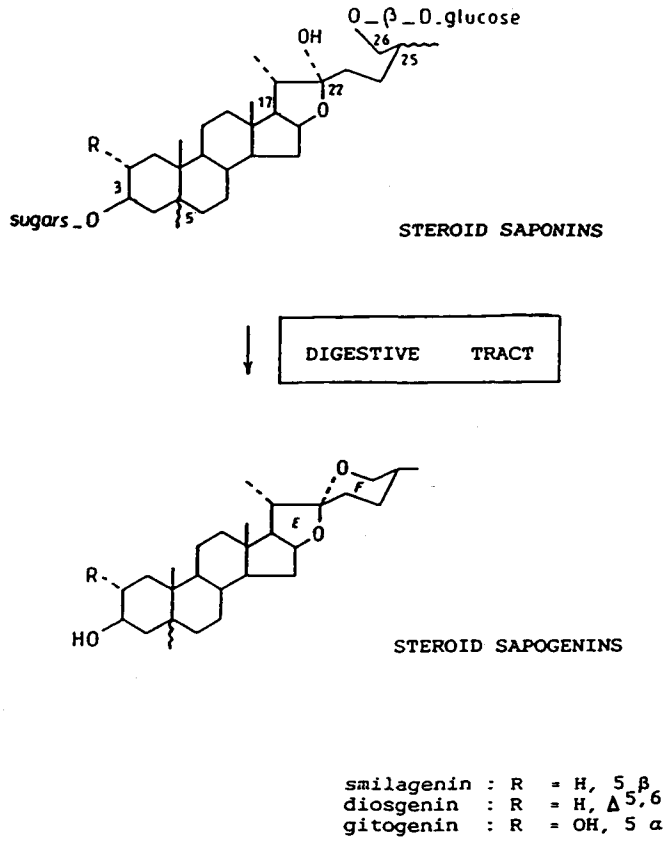

FIG. 1. Transformation of steroid saponins from fenugreek in the digestive tract.

Furthermore, Malinow (13) has shown that diosgenin glucoside was more efficient in reducing intestinal absorption of cholesterol than diosgenin. At comparable small doses, diosgenin glucoside inhibited cholesterol absorption, whereas diosgenin did not (13,14). Saponins and/or diosgenin could hence be involved in bringing about the hypocholesterolemic effect of fenugreek seeds. It was thus important to study the transformation of fenugreek subfractions upon their passage through the digestive tract. Toward this aim, we have carried out a study in alloxan-diabetic dogs.

MATERIALS AND METHODS

Plant material. Fenugreek seeds (cultivar Gouka) were obtained from our experimental field in Montpellier, France (Université des Sciences et Techniques du Languedoc).

The defatted fenugreek seeds were obtained as previously described (15) and were further fractionated to obtain subfractions enriched in saponins and pro-

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Abbreviations: FAE, feces alcoholic extract; FLE, feces lipid extract; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

tein. The defatted seeds were fractionated into two parts—a fraction containing testa plus endosperm (Subfraction A) and a fraction containing cotyledons plus axis (Subfraction B). The latter was extracted with isopropanol/water (70:30, v/v). The lyophylized extract contained practically all the saponins (Subfraction S), but not the sapogenins (aglycone part of saponins). The insoluble portion contained protein without saponin (Subfraction P).

The techniques used to determine the composition of the subfractions of defatted fenugreek seeds have been previously described (16). Subfraction B includes 7.2% saponins and 52.8% protein; Subfraction S contains 22.2% saponins without protein; Subfraction P contains 70.5% protein without saponins.

Alloxan-diabetic dogs. Conscious mongrel male dogs, weighing 18–21 kg, were made diabetic by an intravenous injection of alloxan (50 mg/kg) at least 45 days before the experiment. The animals were housed in individual cages. They were fed twice daily with a standard diet (30 g/kg at each meal, in the form of patties containing 9% protein, 5% lipid and 78% moisture; UAR, Epinay, France).

Each animal received two daily subcutaneous injections (14–24 IU) of soluble insulin (Endopancrine, Organon, France) so that they were clearly glycosuric (about 50 g/24 hr) and had high plasma levels of cholesterol, but they were not ketotic. The dose of insulin injected was kept constant throughout the experiment.

Experimental protocols. Fenugreek seed subfractions were administered with the animals diet for 21 days. Four sets of experiments were performed: i) Four dogs received Subfraction B at 1.13 g/kg corresponding to 0.08 g/kg per day of saponins and 0.59 g/kg per day of protein; ii) four dogs received Subfraction S at 0.37 g/kg corresponding to the same dose of saponins—0.08 g/kg per day; iii) four dogs received Subfraction P at 0.73 g/kg corresponding to 0.51 g/kg per day of protein; and iv) four dogs were fed the standard diet (control experiments). The doses were chosen so that the animals received an amount of saponins (Subfractions B and S) or protein (Subfraction P) corresponding to those of the total defatted fraction previously used (3).

Feces from each dog were collected before and during the treatments and the composition of fecal saponins or sapogenins was determined. Simultaneously, the effects on plasma cholesterol levels were evaluated, according to the colorimetric method of Watson (17), using blood samples collected from jugular vein.

Fecal saponin analysis. Feces were immediately sampled and freeze-dried; 5–15 g were ground in a mortar and extracted in a Soxhlet apparatus overnight with hexane under reflux. The hexane extract was called feces lipid extract (FLE). The defatted fecal samples were reextracted in a Soxhlet apparatus overnight with isopropanol/water (70:30, v/v). The alcoholic extract was called feces alcoholic extract (FAE).

The steroid sapogenins in FLE were characterized by thin-layer chromatography (TLC) on silica gel (precoated silica gel TLC plates, Merck, Darmstadt, Germany) developed with hexane/ethyl acetate (60:40, v/v). Fractions were made visible with $SbCl_3$/*6 N* HCL (1:1, w/v) or with methanol/H_2SO_4 (1:1, v/v) followed by heating.

To isolate the steroid sapogenins, the FLE was applied as band onto TLC plates and the plates were developed in hexane/ethyl acetate (40:60, v/v). The sapogenins were separated into an upper zone (R_f, 0.7–0.8), consisting of monohydroxylated sapogenin that co-developed with standard diosgenin, and a bottom zone (R_f, 0.2–0.3), consisting of dihydroxylated sapogenins that co-developed with standard gitogenin [(25 R)-5α-spirostan-2,3β-diol]. Silica gel from the two zones was scraped from the plates and eluted with a series of solvents, namely hexane, dichloromethane, and then ethanol. Five successive preparative TLC fractionations were necessary to obtain relatively pure fractions, as judged by GC.

The monohydroxy sapogenins recovered from the last TLC separation were further purified by reversed-phase high performance liquid chromatography (HPLC) (C18 column Ultrasphere ODS, 5 μdp, 4.6 mm i.d. × 250 mm) eluted with methanol/water (96:4, v/v). The flow rate was set at 1.6 mL/min, and the detector at 205 nm. Diosgenin was eluted in 7.5 min, cholesterol in 24 min, and sitosterol was eluted in 32.5 min. Monohydroxy sapogenins (like diosgenin) were collected after repeated injection onto the column. The identity of each isolated mono or dihydroxy sapogenin was established by a comparison of retention times in GC and of mass spectra (GC/MS) with those of standards.

Capillary gas chromatography (GC). A Delsi gas chromatograph (model D 1700) equipped with a FID and a Shimadzu integrator (model C-R4A, Shimadzu, Kyoto, Japan) were used. The chromatograph was fitted with a RSL 150 (Alltech, Deerfield, IL) 30-m fused silica capillary column of 0.25 mm i.d. bound and cross-linked with a polydimethylsiloxane film 0.2 μm thick. The analyses were run isothermally at 270°C; the injector and detector temperatures were 300°C. Hydrogen was used as carrier gas with a head pressure of 0.7 bar (10 psi).

Capillary gas chromatography/mass spectrometry (GC/MS). GC/MS was carried out with an LKB Bromma 209 equipped with Vg Digital pdp 8a computer. The gas chromatograph was fitted with a capillary column OV 1701 (25 m, 0.32 mm i.d., 0.20 μm film). Analyses were carried out isothermally at 260°C for monohydroxy sapogenins and at 290°C for dihydroxy sapogenins. Helium was used as carrier gas with a head pressure of 0.5 bar (7 psi). The jet separator was maintained at 230°C. Ionizer temperature was set at 250°C and electron energy and electron multiple voltage were 70 eV and 3.5 kV, respectively. Smilagenin [(25 R)-5β-spirostan-3β-ol] was from Merck, and diosgenin was from K and K Laboratories Inc. (Plainview, CA). Gitogenin was isolated from *Trigonella foenum graecum* and characterized by GC/MS and ^{1}H nuclear magnetic resonance (NMR).

RESULTS

Total food intake was kept the same in the different experimental groups. The data in Table 1 show that subfractions B and S induced a significant reduction

TABLE 1

Effect of Chronic Intake of Fenugreek Seed Subfractions Combined with Insulin Treatment on Plasma Cholesterol Levels (mg/100 mL) in Alloxan Diabetic Dogs[a]

	Days/Insulin treatment[b]										
				Fenugreek intake							
	Day −42	−40	−2	−1	1	6	9	14	17	21	28	33
Fenugreek Subfraction B (4)	80 ± 15		238 ± 7	246 ± 6	215[c] ± 14	189[c] ± 14	199[c] ± 20	200[c] ± 3	193[c] ± 9	202 ± 11	230 ± 20	241 ± 10
Fenugreek Subfraction S (4)	89 ± 12		257 ± 16	256 ± 19	233 ± 17	229 ± 9	226[d] ± 12	206[d] ± 11	192[d] ± 18	203 ± 13	229 ± 14	239 ± 15
Fenugreek Subfraction P (4)	92 ± 13		259 ± 5	268 ± 8	276 ± 8	277 ± 7	274 ± 8	270 ± 10	279 ± 12	286 ± 13	296 ± 21	292 ± 20
Controls (4)	68 ± 10		248 ± 21	263 ± 13	266 ± 18	268 ± 20	267 ± 20	283 ± 17	274 ± 21	272 ± 22	277 ± 19	281 ± 21

[a]Results are expressed as mean ± SEM. The number of animals is indicated in parentheses. Subfraction B (saponins, 7.2%; proteins, 52.8%). Subfraction S (saponins, 22.2%). Subfraction P (proteins, 70.5%).
[b]Alloxan was injected on day −41.
[c]$p<0.01$.
[d]$p<0.05$.

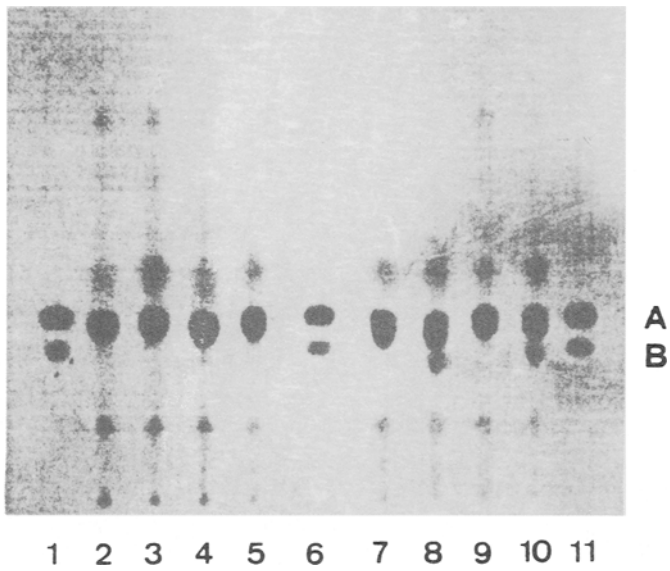

FIG. 2. Thin-layer chromatogram of feces lipid extracts (FLE) from dogs receiving Subfractions P and S of fenugreek. Lanes 1, 6, 11: Cholesterol (R_f, 0.37) plus diosgenin (R_f, 0.30). Lanes 2, 3, 4, 5: FLE from dogs receiving Subfraction P. Lanes 2, 4: Before treatment. Lanes 3, 5: After treatment. Lanes 7, 8, 9, 10: FLE from dogs receiving Subfraction S. Lanes 7, 9: Before treatment Lanes 8, 10: After treatment. A indicates the presence of substances with an R_f equal to that of cholesterol. B indicates the presence of substances with an R_f equal to that of diosgenin.

in plasma cholesterol in diabetic dogs; in contrast, Subfraction P had no effect.

Figure 2 shows a thin-layer chromatogram obtained with FLE from feces of dogs receiving either Subfraction S or Subfraction P. TLC clearly reveals the appearance of a new substance, but only in the feces from dogs given Subfraction S. The same substance was also detected in FLE from dogs given Subfraction B (saponins 7.2%) (results not shown). These chromatographic properties (R_f, chromophore) of the substance suggested that it belonged to the class of monohydroxylated sapogenins, such as diosgenin. Nevertheless, the results obtained by TLC needed to be confirmed by complementary methods.

After purification by preparative TLC and HPLC, several samples were analyzed by GC and GC/MS. The chromatogram of monohydroxy steroid sapogenins upon capillary GC shows three principal peaks, as well as several minor ones (Fig. 3). The retention times of peaks No. 1 and No. 2 correspond to smilagenin and diosgenin, respectively. For further confirmation, we analyzed the samples by capillary GC/MS. The chromatogram exhibits the same pattern as the two principal peaks 1 and 2 (Fig. 3A). The ionogram obtained by ion selective monitoring at *m/z* 139 is practically identical to the ion chromatogram in Figure 3B. It seems likely that the peak at *m/z* 139 $[C_9H_{15}O]^+$ arises from ring E fragmentation by a 1,2 shift of the C-20 hydrogen to C-17 (Fig. 1). The fragment is characteristic of a steroid sapogenin with a spiroketal side chain (18,19).

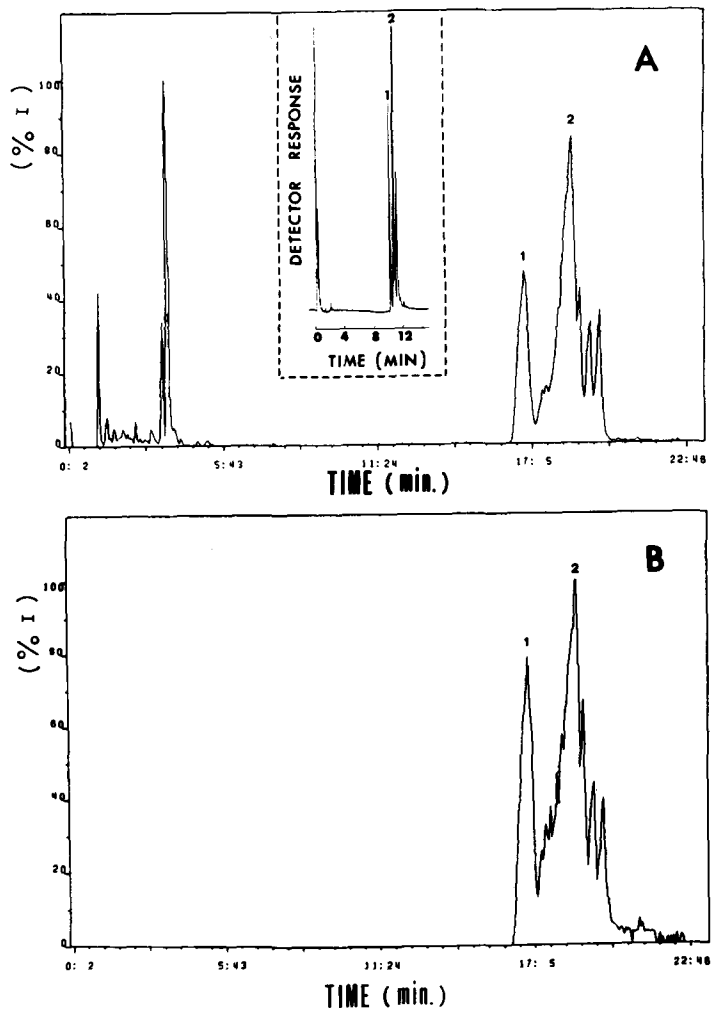

FIG. 3. Capillary GC/MS of isolated monohydroxy steroid sapogenins from feces of dogs receiving steroid saponins. A: Ion electron impact chromatogram (capillary GC). B: Ion current profile of selected ion *m/z* 139.

The mass spectrum of the substance corresponding to peak No. 1 reveals a molecular peak at *m/z* 416 which corresponds to the mass of smilagenin (Fig. 4A). Likewise, the mass spectrum of the substance corresponding to peak No. 2 shows a molecular peak at *m/z* 414 which corresponds to the mass of diosgenin (Fig. 4B).

The other fragmentations observed allowed us to confirm our assignments (Figs. 4A and 4B). Fission of the F ring produces ions $[M\text{-}C_4H_8O]^+$ at *m/z* 344 or 342. Corresponding to $[C_{23}H_{36}O_2]^+$ or $[C_{23}H_{34}O_2]^+$, and ions $[M-C_3H_7O]^+$ at *m/z* 357 or 355. Corresponding to $[C_{24}H_{37}O_2]^+$ or $[C_{24}H_{35}O_2]^+$, in the smilagenin or diosgenin spectra, respectively. Another group of characteristic ions arises by fragmentation in ring E, producing ions at *m/z* 302 or 300 corresponding to $[C_{21}H_{34}O]^+$ or $[C_{21}H_{32}O]^+$ in the smilagenin or diosgenin spectra, respectively. Other characteristic ion peaks occurred at *m/z* 255 (253), 269 (267), and 284 (282) due to hydrocarbon fragments. Standards of smilagenin and diosgenin showed spectra and fragmentation patterns very similar to those of peaks No. 1 and 2, respectively.

The chromatogram of dihydroxy steroid sapogenins obtained upon capillary GC shows several peaks and two principal ones, Nos. 3 and 4 (Fig. 5). The retention time of peak No. 3 corresponds to that of gitogenin. The assignment was confirmed by capillary GC/MS (Fig. 5A). The ionogram obtained by ion selective monitoring at *m/z* 139 is practically identical to the ion chromatogram in Figure 5B. The mass spectrum (Fig. 4C) exhibits a molecular peak at *m/z* 432 corresponding to the mass of gitogenin. The spectrum also shows prominent ions at *m/z* 360 $[C_{23}H_{36}O_3]^+$, *m/z* 363 $[C_{22}H_{35}O_4]^+$, and *m/z* 373 $[C_{24}H_{37}O_3]^+$. Another group of characteristic ions arises from cleavage at the spiroketal site. Ions *m/z* 318 indicates that the additional hydroxy group in gitogenin is nuclear. A standard of gitogenin showed a spectrum and fragmentation pattern very similar to those of

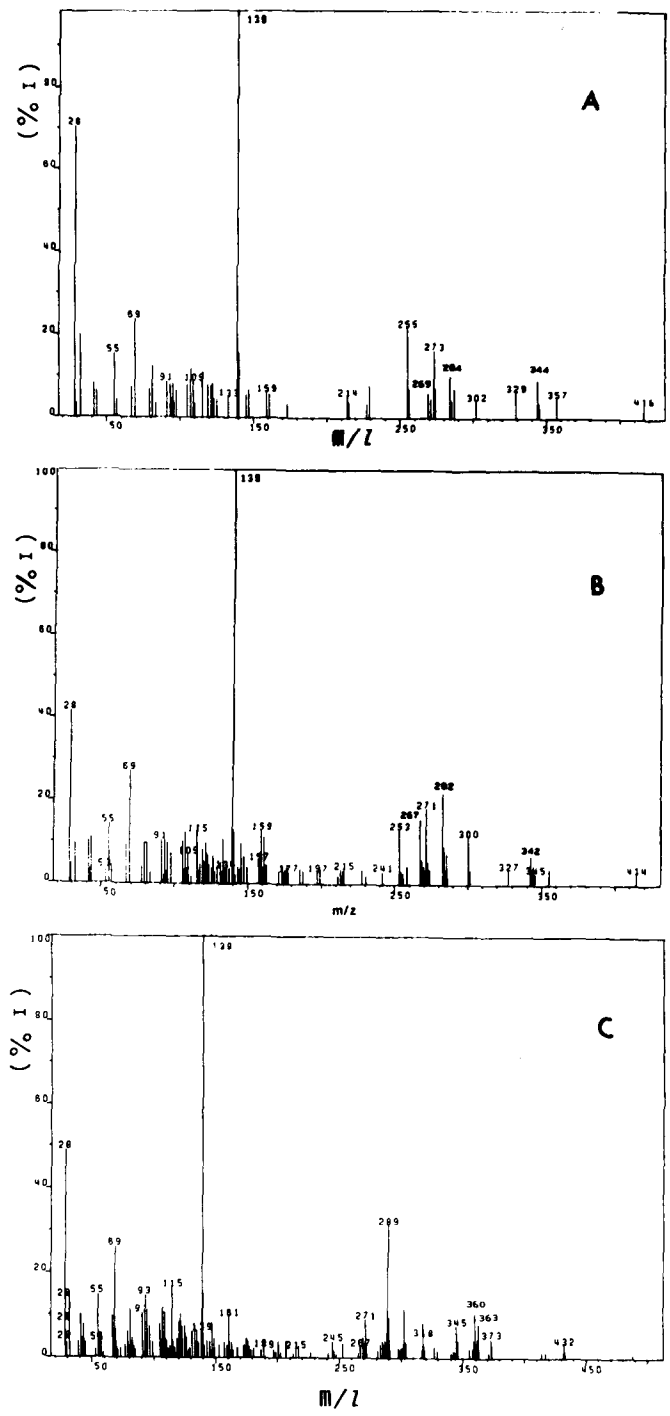

FIG. 4. Mass spectra of isolated steroid sapogenins from feces of dogs receiving steroid saponins. A: Mass spectrum of peak No. 1. B: Mass spectrum of peak No. 2. C: Mass spectrum of peak No. 3.

peak No. 3. Peak No. 4 has not been identified so far.

Assays of sapogenins either in the free form (in FLE) or in the glycosylated form (in FAE) were carried out to determine the extent of hydrolysis of steroid saponins in the digestive tract. The results obtained from six different feces samples indicated that 57.4 ± 5.3% (SD) of the saponins were hydrolyzed to sapogenins.

We also investigated *in vitro* whether β-glucosidase could hydrolyze a sample of fenugreek saponins to the sapogenins. For this purpose, the purified S subfraction was subjected to β-glucosidase digestion for 24 hr. The results showed that no sapogenin was released by this treatment, indicating that β-glucosidase is ineffective in cleaving the glycoside bond at C-3 (Fig. 1). However, the enzyme acted on the saponins releasing the glucose residue from C-26. The native saponins of the furostanic type were then converted to saponins of the spirostanic type. We also calculated the diosgenin/smilagenin ratio based on chromatographic analysis (GC). The ratio was 19 for the S subfraction before ingestion, and 2 for FLE.

DISCUSSION

Our study showed that fenugreek subfractions rich in steroid saponins are, in part, transformed in the gastrointestinal tract. Thus, smilagenin, diosgenin and gitogenin were detected in feces of dogs fed fenugreek (Fig. 1). Hydrolysis averaged 57%. The 25 S epimers corresponding to smilagenin, diosgenin and gitogenin, as well as their saturated or unsaturated derivatives, may also be present in the feces.

The location of fenugreek saponin hydrolysis in the digestive tract has not been determined. If sapogenins are able to partially prevent cholesterol absorption, saponin hydrolysis presumably occurs in the stomach or/and in the proximal small intestine. However, Gestetner *et al.* (20) had previously reported that ingested soybean saponins are hydrolyzed by cecal microflora in chicks, rats and mice. The apparent discrepancy may be due to differences in species or other experimental conditions.

Calculation of the diosgenin/smilagenin ratio revealed a clear difference between the composition of Subfraction S and the lipid extract obtained from the feces (19 *vs* 2). This greater amount of smilagenin in the feces seems to be due to transformation of the sapogenins from Subfraction S during their passage through the gastrointestinal tract.

Previous reports have shown that other plant steroids with a double bond in 5,6-position are reduced and transformed into 5β-hydroxy steroid analogues by gastrointestinal microorganisms in various animals (21,22). It is possible that in our experiments diosgenin was reduced and transformed to smilagenin in the digestive tract of the dogs. However, it is important to recall that differences in the biotransformation of diosgenin have been observed depending on the species studied and the conditions of administration employed (23).

As to the hypocholesterolemic effect of fenugreek seeds, the presence of steroid saponins seems to play an essential role (4). Other saponins have also been reported to modify cholesterol metabolism. Thus, alfalfa saponins, as well as digitonin, inhibited cholesterol intestinal absorption in the monkey (7,24). Soybean saponins reduced intestinal uptake of cholesterol

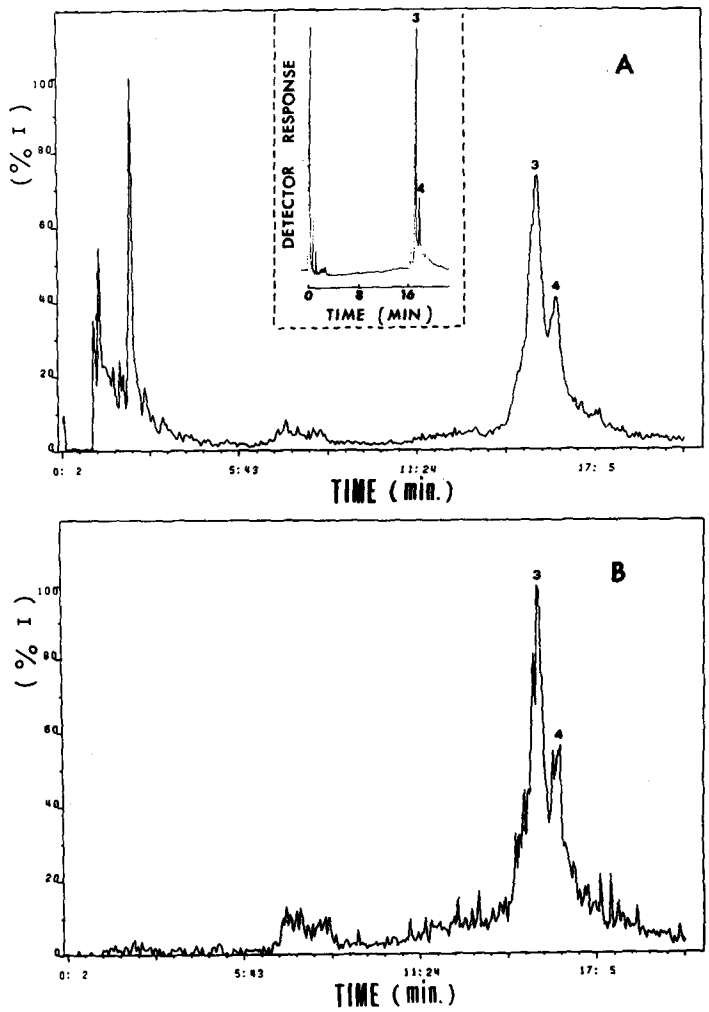

FIG. 5. Capillary GC/MS of isolated dihydroxy steroid sapogenins from feces of dogs receiving steroid saponins. A: Ion electron impact chromatogram (capillary GC). B: Ion current profile of selected ion *m/z* 139.

bean saponins reduced intestinal uptake of cholesterol in the rat (6). It appears likely that saponins and cholesterol form insoluble complexes (25–27) which may retard cholesterol absorption in the intestine. On the other hand, certain saponins from soapwort (*Saponaria officinalis L*), soybeans, and quillaya (*Quillaya saponaria*) also form large mixed micelles with bile acids (28). The increase in fecal excretion of bile acids and neutral steroids could thus be compensated for by an increased conversion of cholesterol to bile acids by the liver. Furthermore, in the rat fenugreek induced an increase in bile acid output (2,29), which suggests increased transformation of cholesterol into bile acids by the liver. This could contribute to the hypocholesterolemic activity of fenugreek.

Our results show that the ingested fenugreek saponins are partially hydrolyzed to diosgenin in the gastrointestinal tract. Other steroid sapogenins (smilagenin, gitogenin) were also found in the feces of the dogs. It is known that diosgenin interferes with the absorption of cholesterol (10). The decreased cholesterol absorption associated with enhanced secretion of cholesterol into bile resulted in increased excretion of neutral sterols (10,12).

Our results suggest that the hypocholesterolemic effect of fenugreek seeds might be due to saponins and that diosgenin produced by gastrointestinal hydrolysis may contribute to this effect.

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