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Comparative pharmacokinetics of silipide and silymarin in rats

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SUMMARY

The plasma level profile and the biliary excretion of silybin, the main flavanolignan component of silymarin, were evaluated in rats after single equimolar oral doses (200 mg/kg, expressed as silybin equivalents) of the silybin-phosphatidylcholine complex silipide (laboratory code IdB 1016) and of silymarin. Silybin was assayed by using a specific HPLC method which allowed also the determination of other flavanolignans present in the biological fluids after administration of silymarin (i.e. silydianin, silycristin and isosilybin).

After oral silipide, silybin reached peak plasma levels within 2 h, with a C_{max} of 9.0 ± 3.0 μg/ml for unconjugated drug and 93.4 ± 16.7 μg/ml for total (free + unconjugated drug). Maximum total biliary concentrations of silybin (2989 ± 568 μg/ml) were observed within 2 h and the biliary recovery after 24 h accounted for about 13% of the administered amount.

After administration of silymarin, unconjugated and total plasma silybin levels as well as biliary excretion were several-fold lower than those observed after treatment with silipide. Silybin recovered over a 24 h period after silymarin intake accounted for about 2% of the administered dose.

Plasma and bile obtained after administration of silymarin contained also silydianin, silycristin and, to a greater extent, isosilybin. The concentrations of the latter compound in plasma and in bile were higher than those of silybin itself.

The relative bioavailability of silipide (calculated in the target organ as the ratio between AUCs of cumulative biliary excretion curves) was 10-fold higher than that of silymarin.

INTRODUCTION

Silymarin, a standardized extract of flavanolignans from *Silybum marianum* seeds, is widely used in Europe for the treatment of liver disorders (1–3). Silybin is the main constituent of silymarin, but silycristin, silydianin and isosilybin are also present in the extract.

A problem associated with the oral use of silybin is represented by the low bioavailability of this compound, as demonstrated by studies in laboratory animals (4, 5) and in man (6).

The in vitro absorbability of silybin can be greatly improved (7) by its chemical complexation with phosphatidylcholine in order to obtain the very lipophilic compound silipide (8). Increased lipophilicity accounts for the enhanced oral absorption of silybin when administered as silipide in rats (4). Adequate bioavailability, in turn, accounts for the dose-related oral activity of silipide in several experimental models of liver intoxication (9).

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Studies in rats (10), in healthy volunteers (11) and in patients with liver disease (12, 13) have demonstrated that after oral intake of silipide, plasma silybin levels are several-fold higher than those measured after treatment with silymarin at doses equimolar in terms of silybin content.

Since the liver represents the target organ for silybin, it was considered of interest to compare the biliary excretion and plasma levels profile of the flavanolignan after single oral equimolar (200 mg/kg as silybin) doses of silipide and silymarin in rats. The dose of silipide selected for this study is close to the oral ED_{50} calculated in experimental models of liver intoxication.

A modification of a sensitive and specific HPLC assay recently described (14) allowed the simultaneous determination of silybin and of the other silymarin constituents silydianin, silycristin and isosilybin in the plasma and bile of silymarin-treated animals.

Since the metabolism of silybin is reported to be almost exclusively conjugative (15), all bile samples were hydrolyzed with β-glucuronidase/arylsulfatase in order to evaluate the total biliary excretion of the administered flavanolignans.

In plasma, silybin concentrations were determined separately with and without enzymatic hydrolysis, in order to differentiate between free (unconjugated) and conjugated drug.

Part of this study has been presented at 'Quatrièmes Journées Méditerranéennes de Pharmacocinétique'. Montpellier, 28–30 March 1991.

MATERIALS AND METHODS

Chemicals

Silybin, silydianin, silycristin and isosilybin were isolated from silymarin by column chromatography and purified by crystallization from methanol. Silipide was obtained by complexation of silybin with phosphatidylcholine and characterized by spectroscopic analysis (8). Silymarin was from Indena (Inverni della Beffa Group), eriodictyol from Extrasynthèse (Génay, France), nicardipine from Sigma (St Louis, MO, USA). β-glucuronidase/arylsulfatase was purchased from Boehringer Mannheim (Mannheim, Germany). n-Hexane, methanol, ethanol, tert-butylmethylether, acetate buffer, citrate buffer and Extrelut® columns were supplied by Merck (Darmstadt, Germany).

Animals and sample preparation

Plasma levels

Validation curves : 3 male Sprague-Dawley rats (200–220 g), fasted overnight, were used. Blood was collected, under ether anaesthesia, from the abdominal aorta into heparinized tubes and centrifuged at 2500 rpm for 20 min in order to obtain blank plasma. 10 µl of methanol containing increasing amounts of silybin, silydianin, silycristin and isosilybin were evaporated under nitrogen and resuspended with 50 µl of pooled blank plasma. These samples were then frozen and stored at –20°C until analyzed. The concentrations used (run in triplicate) were 0.25, 0.5, 2.5, 5, 10, 20 µg/ml for unconjugated silybin and 3.12, 6.25, 12.5, 25, 50, 100 µg/ml for total silybin; 0.2, 0.8, 3.2 µg/ml for unconjugated flavanolignans and 1.56, 3.12, 6.25, 12.5, 25, 50 µg/ml for total flavanolignans.

Treatment of animals and collection of samples : 12 male Sprague-Dawley rats (200–220 g) were randomly assigned to 2 groups of 6 animals each and chronically cannulated through the jugular vein under ether anaesthesia as previously described (4). The animals were fasted overnight and then treated with single oral doses of silipide (200 mg/kg as silybin; n = 6) or silymarin (200 mg/kg as silybin; n = 6). Blood samples (350 µl) were obtained in heparinized tubes at 0, 0.25, 1, 2, 4, 6, 8 and 24 h after administration. After centrifugation at 2500 rpm for 20 min the plasma was immediately frozen and stored at –20°C until analyzed.

Extraction procedures : For the determination of free (unconjugated) flavanolignans, thawed plasma (50 µl) was mixed with 2.9 ml of citrate buffer (pH 4), placed on Extrelut 3 and eluted twice with 10 ml of tert-butylmethylether.

For the determination of total (unconjugated + conjugated) flavanolignans, thawed plasma (50 µl) was mixed with 150 µl of acetate buffer (pH 5), 5 µl of HCl (1 N) and 10 µl of β-glucuronidase/arylsulfatase (about 0.055 U of glucuronidase and 0.026 U of arylsulfatase activity). After incubation at 37°C for 48 h, 2.7 ml of citrate buffer (pH 4) was added. Samples were then placed on Extrelut 3 and eluted as above.

Eluates were evaporated under nitrogen and the residue was resuspended in 100 µl of mobile phase. After addition of 5 or 10 µl of a diluted methanol solution of eriodictyol (internal standard; 0.2 mg/ml), samples were analyzed for flavanolignan content.

Frozen samples for validation curves, prepared as

described previously, were thawed at room temperature, extracted and analyzed as described above for their flavanolignan content. Daily standards were prepared by adding to test tubes 10 µl of methanol containing increasing amounts of silybin, silydianin, silycristin and isosilybin. After evaporation under nitrogen, the residues were resuspended with 50 µl of pooled blank plasma and processed as described above in parallel with samples obtained from treated rats. For the assay of samples collected after silipide treatment, the range of concentrations of the standards was 0.25–20 µg/ml for unconjugated silybin and 3.12–200 µg/ml for total silybin. For the assay of the samples collected after silymarin treatment, the range of concentrations of the standards was 0.2–3.2 µg/ml for unconjugated flavanolignans and 1.56–50 µg/ml for total flavanolignans.

Biliary excretion

Validation curves : 3 male Sprague-Dawley rats (about 300 g), fasted overnight, were cannulated at the bile duct (16) under urethane anaesthesia (25% urethane aqueous solution; 5 ml/kg i.p.). Blank bile was collected over 6 h and then pooled. 100 µl of methanol containing increasing amounts of silybin, silydianin, silycristin and isosilybin was evaporated under nitrogen and resuspended with 250 µl of pooled blank bile. Samples were frozen and stored at –20°C until analyzed. The concentrations used (run in triplicate) were 10, 100 and 500 µg/ml.

Treatment of animals and collection of samples : 12 male Sprague-Dawley rats (about 300 g) were randomly assigned to 2 groups of 6 animals each and fasted overnight. The animals were cannulated at the bile duct under urethane anaesthesia (25% urethane aqueous solution; 5 ml/kg i.p.) and then treated with single oral doses of silipide (200 mg/kg as silybin; n = 6) or silymarin (200 mg/kg as silybin; n = 6). After treatment, bile was collected during the following intervals: 0–2, 2–4, 4–6, 6–8, and 8–24 h (a blank sample was also obtained before drug administration). After collection, all samples were frozen immediately and stored at –20°C until analyzed.

Extraction procedures : Thawed bile (0.25 or 0.025 ml) was mixed with 0.75 ml of acetate buffer (pH 5) and 50 µl of β-glucuronidase/arylsulfatase. After incubation at 37°C for 24 h, samples were placed on Extrelut 1 and eluted twice with 4 ml of tert-butylmethylether. The eluates were evaporated under nitrogen and the residue was resuspended in 200 µl (500 µl for samples collected after silipide treatment) of mobile phase. After addition of 10 µl (25 µl for samples collected after silipide treatment) of a methanol solution of nicardipine (internal standard; 1 mg/ml), the samples were analyzed for flavanolignan content.

Frozen bile samples for validation curves, prepared as described previously, were thawed at room temperature and then extracted and analyzed as described above for their content in total flavanolignans.

Identical amounts of 4 methanolic solutions of flavanolignans were added to test tubes to obtain samples for recovery calculations. After evaporation under nitrogen the residue was resuspended as above.

Daily standards were prepared by adding to test tubes 100 µl of methanol containing increasing amounts of silybin, silydianin, silycristin and isosilybin. After evaporation under nitrogen, the residues were resuspended with 250 µl of pooled blank bile processed as above in parallel with samples obtained from treated rats and finally resuspended in 200 µl of mobile phase and 10 µl of internal standard (ISTD). The range of concentrations was 10–500 µg/ml.

HPLC

Silybin, silydianin, silycristin and isosilybin were determined in plasma and in bile by HPLC. Analyses were carried out on a Waters System equipped with a WISP 700 Automatic autosampler, a Waters 600 E System control and a Waters 600 Multidelivery System; the detection at 214 nm was accomplished by using a Model L-4200 UV-VIS (Hitachi). Two LiChrosorb Diol 5 µm columns (150 mm x 3 mm; E. Merck, Darmstadt, Germany) were used. The mobile phase was n-hexane/ethanol 80:20 containing 2 g/l of tetrabutylammonium hydrogensulfate and 120 µl of H_3PO_4 85% at a flow rate 1.2 ml/min. The injected volume was 20 µl.

Data analysis

Areas under the plasma concentration-time curve (AUC_{0-24h}) were calculated by the trapezoidal rule. C_{max} and T_{max} were derived directly from experimental data.

Bile AUC_{0-24h} values (%Dose.h) were calculated by the trapezoidal rule from individual cumulative excretion curves. C_{max} and T_{max} were derived directly from experimental data.

The relative bioavailability of silybin was esti-

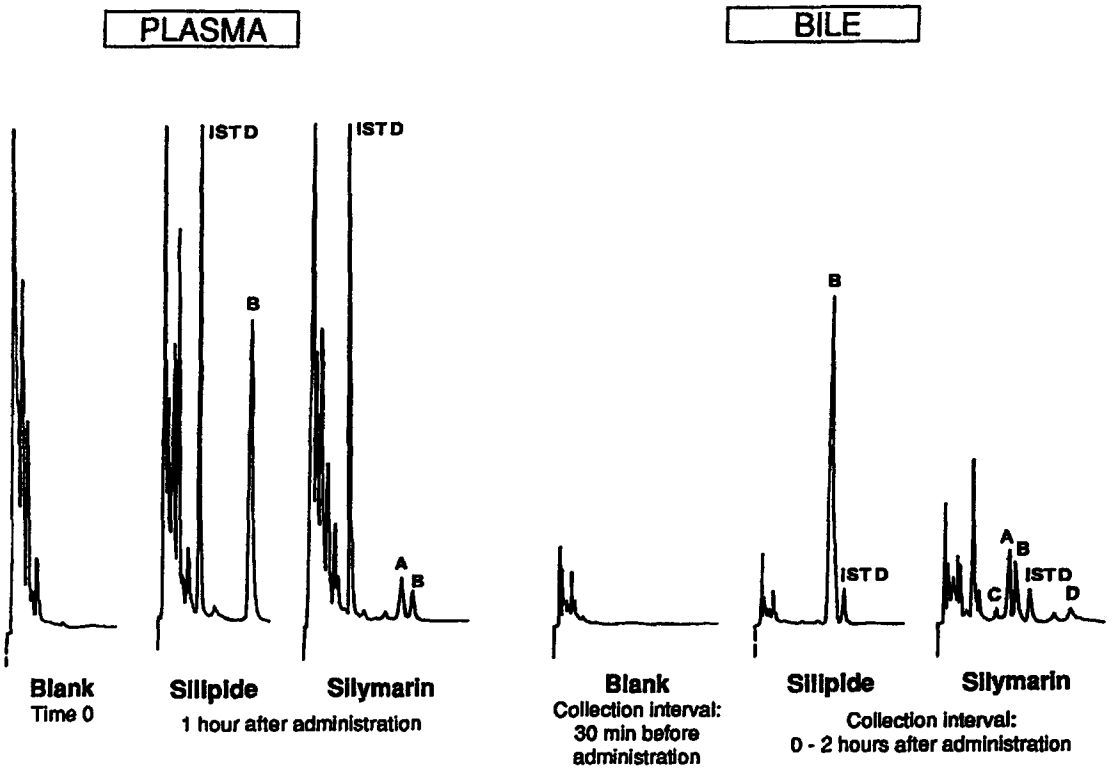

Fig. 1 : Representative HPLC chromatograms of flavanolignans in rat plasma and bile. Silipide and silymarin were administered orally at the dose of 200 mg/kg (as silybin). ISTD = internal standard; A = isosilybin; B = silybin; C = silydianin; D = silycristin. Final dilution ratio (before HPLC analysis) of bile samples was 2.5 (silipide) : 1 (silymarin)

mated by calculating the ratio of mean AUCs in bile.

Data are expressed in the text as means ± SE.

RESULTS

HPLC profiles of flavanolignans

The HPLC method allowed a good separation of silybin, isosilybin, silydianin and silycristin in plasma and bile (Fig. 1).

Plasma

Validation of the analytical method

Standard curves for unconjugated flavanolignans were linear. Precision and accuracy data are shown in Table I.

The lowest concentration value (0.25 µg/ml for silybin and 0.2 µg/ml for other flavanolignans) was considered as the limit of quantitation. Recovery was found to be quantitative. Standard curves for total flavanolignans were linear. Precision and accuracy data are shown in Table II.

The lowest concentration value (3.12 µg/ml for silybin and 1.56 µg/ml for other flavanolignans) was considered as the limit of quantitation. Recovery was found to be quantitative.

Plasma level profiles of flavanolignans

Mean plasma levels of unconjugated and total flavanolignans after administration of silipide or silymarin are shown in Figures 2 and 3 respectively. Pharmacokinetic parameters are reported in Table 3.

After administration of silipide, a well defined plasma level profile of unconjugated silybin was observed. C_{max} values ranged from 1.1 to 20.1 µg/ml with a mean value of 9.0 ± 3.0 µg/ml. AUCs were in the 1.1–16.3 h.µg/ml range, with a mean value of 8.3 ± 2.4 h.µg/ml. T_{max} ranged from 0.25 to 1.00 h. Most of the silybin in plasma was in conjugated form. After hydrolysis, C_{max} averaged 93.4 ± 16.7 µg/ml

Table I: Precision and accuracy of the assay of unconjugated silymarin flavanolignans in rat plasma

Flavanolignan	*Actual concentration (μg/ml)*	*Calculated concentration (mean values; n = 3) (μg/ml)*	*Precision (%)*	*Accuracy (%)*
Silybin	0.25	0.26	12.3	+4.0
	0.50	0.45	18.5	–9.4
	2.50	2.80	34.7	+11.5
	5.00	4.50	6.6	–10.0
	10.00	9.30	8.1	–7.4
	20.00	20.70	2.3	+3.5
Isosilybin	0.20	0.20	19.8	+2.0
	0.80	0.79	3.8	–0.8
	3.20	3.20	3.8	0.0
Silydianin	0.20	0.21	14.9	+7.0
	0.80	0.79	40.3	–1.5
	3.20	3.20	19.8	+0.1
Silycristin	0.20	0.22	25.0	+8.5
	0.80	0.78	25.1	–2.6
	3.20	3.20	3.7	+0.1

Table II: Precision and accuracy of the assay of total silymarin flavanolignans in rat plasma

Flavanolignan	*Actual concentration (μg/ml)*	*Calculated concentration (mean values; n = 3) (μg/ml)*	*Precision (%)*	*Accuracy (%)*
Silybin	3.12	3.39	27.5	+8.4
	6.25	6.22	3.4	–0.5
	12.50	12.60	3.3	+0.4
	25.00	24.20	15.3	–3.2
	50.00	52.70	9.1	+5.4
	100.00	96.50	8.5	–3.5
Isosilybin	1.56	2.01	6.5	+28.9
	3.12	3.11	18.3	–0.4
	6.25	6.06	7.8	–3.0
	12.50	12.20	5.5	–0.3
	25.00	25.00	2.8	–0.2
	50.00	50.10	7.8	+0.2
Silydianin	1.56	1.94	20.4	–24.4
	3.12	2.75	34.2	–11.9
	6.25	5.72	24.8	–8.5
	12.50	11.80	16.3	–5.3
	25.00	28.20	12.0	+12.8
	50.00	49.20	10.4	–1.6
Silycristin	1.56	1.15	20.0	–26.2
	3.12	3.49	5.7	+11.6
	6.25	6.73	3.7	+7.7
	12.50	11.10	16.2	–11.0
	25.00	25.10	4.2	+0.5
	50.00	50.20	8.7	+0.5

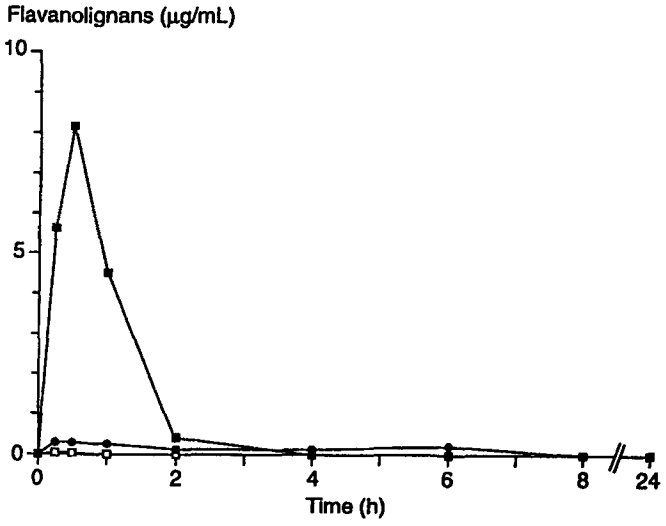

Fig. 2 : Mean plasma levels of unconjugated flavanolignans after a single oral dose of silipide and silymarin (200 mg/kg as silybin) in rats. Filled squares, silybin after silipide; open squares, silybin after silymarin; filled circles, flavanolignans (silybin + isosilybin + silydianin) after silymarin

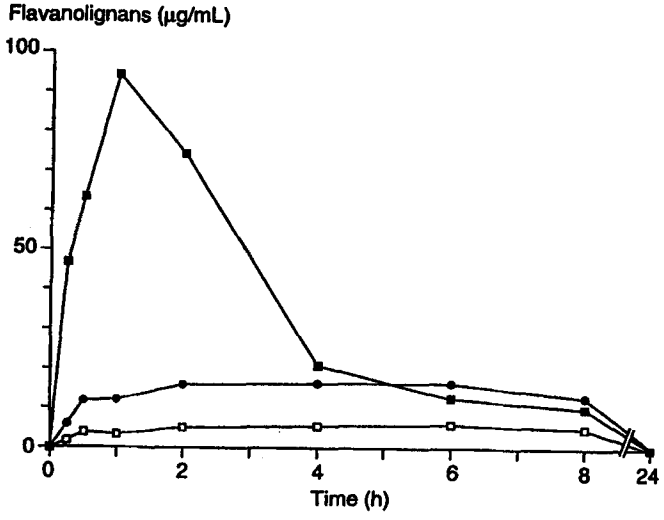

Fig. 3 : Mean plasma levels of total flavanolignans after a single oral dose of silipide and silymarin (200 mg/kg, as silybin) in rats. Filled squares, silybin after silipide; open squares, silybin after silymarin; filled circles, flavanolignans (silybin + isosilybin + silydianin + silycristin) after silymarin

Table III : Pharmacokinetic parameters (means ± SE) derived from plasma unconjugated and total flavanolignan concentrations after a single oral dose of silipide or silymarin (200 mg/kg as silybin) in rats

Silipide			*Silymarin*											
Silybin			*Silybin*			*Isosilybin*			*Silydianin*			*Silycristin*		
C_{max} (µg/ml)	T_{max} (h)	*AUC (0–24h)* (h.µg/ml)	C_{max} (µg/ml)	T_{max} (h)	*AUC (0–24h)* (h.µg/ml)	C_{max} (µg/ml)	T_{max} (h)	*AUC (0–24h)* (h.µg/ml)	C_{max} (µg/ml)	T_{max} (h)	*AUC (0–24h)* (h.µg/ml)	C_{max} (µg/ml)	T_{max} (h)	*AUC (0–24h)* (h.µg/ml)
Unconjugated														
9.02	0.63	8.31	0.06	0.13	0.02	0.44	1.65	1.14	0.10	6.0	0.20	0	0	0
±2.96	±0.13	±2.35	±0.04	±0.09	±0.01	±0.14	±1.09	±0.31	±0.07	±0.0	±0.15			
Total														
93.39	1.04	375.81	6.72	4.25	77.11	11.69	3.9	128.66	0.64	5.0	3.70	3.83	6.6	41.5
±16.72	±0.23	±59.66	±0.78	±1.42	±11.38	±1.87	±1.4	±21.80	±0.45	±3.0	±3.53	±0.51	±3.6	±12.4

and the mean AUC value was 375.8 ± 59.7 h.µg/ml After administration of silymarin, unconjugated silybin was detectable in the plasma of only two out of six rats. Mean C_{max} and AUC values were 0.06 ± 0.04 µg/ml and 0.02 ± 0.01 h.µg/ml respectively. Most of the plasma silybin was in conjugated form (C_{max} = 6.7 ± µg/ml and AUC = 77.1 ± 11.4 h.µg/ml after hydrolysis). Plasma levels of unconjugated and total isosilybin were higher tha those of silybin, mean C_{max} and AUC values being 0.4 ± 0.1 µg/ml and 1.1 ± 0.3 µg/ml and 128.7 ± 21.8 h µg/ml respectively after enzymatic hydrolysis. Unconjugated silydianin was detectable at low levels (0.2 and 0.4 µg/ml) in only 2 out of 6 rats. Unconjugated silycristin was undetectable. After enzymatic hydrolysis the plasma levels of silydianin increased in the two animals with detectable levels of unconjugated compound. By contrast, silycristin was measurable in all hydrolyzed samples, with a mean C_{max} of 3.8 ± 0.5 µg/ml and a mean AUC of 41.5 ± 12.4 h.µg/ml.

Table IV : Precision and accuracy of the assay of total silymarin flavanolignans in rat bile

Flavanolignan	*Actual concentration (μg/ml)*	*Calculated concentration (mean values; n = 3) (μg/ml)*	*Precision (%)*	*Accuracy (%)*	*Recovery (%)*
Silybin	9.82	9.80	2.7	–0.2	
	98.20	105.80	4.8	+7.7	82
	491.00	488.00	1.4	–0.7	
Isosilybin	10.04	10.03	3.6	–0.1	
	100.40	100.30	6.1	–0.1	81
	581.80	500.60	2.0	–0.2	
Silydianin	10.34	10.32	4.8	–0.2	
	103.40	106.40	5.4	+2.9	78
	517.00	517.00	0.0	0.0	
Silycristin	9.94	9.93	7.1	–0.1	
	99.36	99.90	4.9	+0.5	83
	496.80	496.10	2.2	–0.1	

Bile

Validation of the analytical method

Standard curves for total flavanolignans were linear. Precision and accuracy data are shown in Table IV.

10 μg/ml was considered as the limit of quantitation of flavanolignans. Analytical recovery was found to be about 80%.

Biliary excretion of flavanolignans

The volumes of bile collected over the observation period after administration of silipide (13.5 ± 1.1 ml) were comparable to those observed after treatment with silymarin (16.8 ± 1.4 ml). This finding indicates the lack of a differential effect of the two compounds on choleresis.

Mean flavanolignan concentration-time profiles in bile after oral administration of silipide and silymarin are shown in Figure 4.

After intake of silipide, silybin reached a peak level at 2 h and declined rapidly during the subsequent hours.

Cumulative excretion curves (Fig. 5) indicate that about 13% of the administered dose of silybin was excreted within 24 h in bile.

After administration of silymarin, silybin was present in bile at all sampling times but at concentrations several fold lower than those after silipide. The recovery of silybin in bile over a 24 h period after silymarin administration accounted for about 2% of the administered dose.

Pharmacokinetic parameters for silybin biliary excretion after administration of the two preparations are reported in Table V.

Maximum bile concentration, amount excreted in bile and AUC values for cumulative silybin excretion were significantly greater after administration of si-

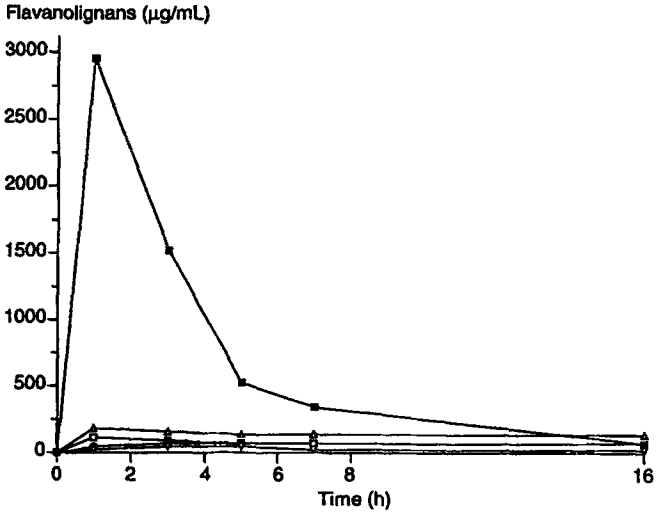

Fig. 4 : Mean biliary concentrations of flavanolignans after a single oral dose of silipide and silymarin (200 mg/kg, as silybin) in rats. Filled squares, silybin after silipide; open squares, silybin after silymarin; open triangles, isosilybin after silymarin; inverted open triangles, silydianin after silymarin; open circles, silycristin after silymarin. Time points are medians of collection intervals

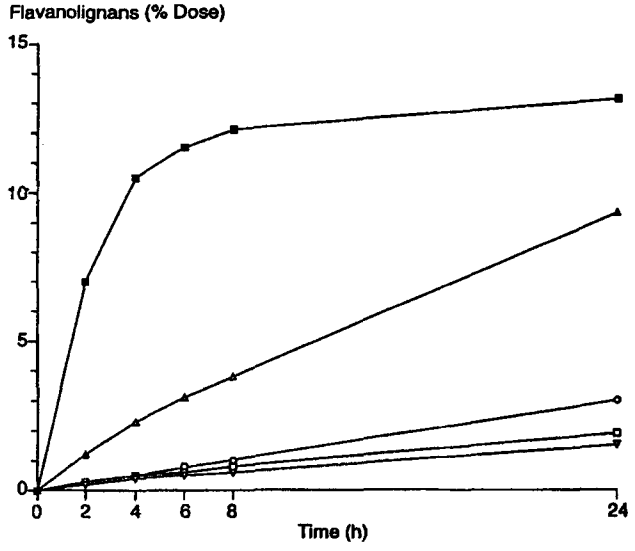

Fig. 5 : Mean cumulative biliary excretion of flavanolignans after a single oral dose of silipide and silymarin (200 mg/kg, as silybin) in rats. Filled squares, silybin after silipide; open squares, silybin after silymarin; open triangles, isosilybin after silymarin; inverted open triangles, silydianin after silymarin; open circles, silycristin after silymarin

lipide than after administration of silymarin. The bioavailability of silybin after the administration of silipide was 10-fold higher than that associated with intake of silymarin.

All samples collected after administration of silymarin contained isosilybin (mean C_{max} = 187 ± 32 µg/ml), silydianin (46 ± 8 µg/ml) and silycristin (85 ± 11 µg/ml). Cumulative excretion curves indicate that the proportion of the administered dose recovered within 24 h was 1.6% for silydianin, 2.9% for silycristin and 9.4% for isosilybin.

Table V : Pharmacokinetic parameters (means ± SE) derived from bile silybin concentrations after a single oral dose of silipide or silymarin (200 mg/kg as silybin) in rats

Parameter	*Silipide*	*Silymarin*
Proportion recovered (0–24h) (% of administered dose)	13.0 ± 3.8**	2.0 ± 0.2
C_{max} (µg/ml)	2989 ± 568**	116 ± 21
T_{max} (h)	1.3 ± 0.4	2.3 ± 1.1
AUC (0–24h) (% dose.h)	271.0 ± 71.0**	26.4 ± 3.2
Relative bioavailability	10	1

**$P < 0.01$, Student's t test.

DISCUSSION

Previous studies in vitro (7) and in vivo after oral administration in rats (4) have demonstrated that the absorbability of silybin can be greatly improved after its complexation with phosphatidylcholine (silipide).

The results of the present investigation confirm the much better bioavailability of silipide compared with silymarin. In particular, these data demonstrate that after oral administration of the complex, the plasma levels and the biliary excretion of silybin are several fold higher than those observed after treatment with an equimolar dose (as silybin) of silymarin.

The biliary excretion of silybin reported after oral administration of silipide in the present study is greater than that recently published by our group (4). This apparent discrepancy could be explained by the different experimental conditions used in the previous study, i.e. low number of animals, collection of bile in unanaesthetized rats, differences in sample hydrolysis and extraction procedures. On the other hand, the present findings on the biliary excretion of silybin are in agreement with the data recently published by Schandalik and co-workers in cholecystectomy patients (13). Since the liver represents the target organ for silipide, the biliary excretion of silybin can be considered as an index of the hepatic uptake of the compound and of its bioavailability at the site of action.

In this respect, our data are in line with those reported previously (15, 17) and demonstrate that, after oral administration of silipide, silybin reaches intracellular sites where it can exert its biological effect (18).

It is of interest that after administration of silipide, plasma and biliary concentrations of silybin are even higher than the sum of the 4 flavanolignans present in the same biological fluids after administration of the extract. Such a comparison, however, may not be fully appropriate. In fact, while silycristin and silydianin are known to retain pharmacological activity, the same may not be true for isosilybin, the most bioavailable of the silymarin flavanolignans. Although pharmacological data on this compound are lacking in the literature, preliminary results from our laboratories indicate that parenterally as well as orally administered isosilybin is inactive in counteracting the lethal effect of phalloidin in mice (unpublished data).

A concluding comment is required about the HPLC method validated in this study, which permitted for the first time the characterization of the plasma and bile levels of the main silymarin flavanolignans in rats. This method represents clearly a major advance and an important tool for pharmacokinetic studies, especially in view of the fact that the specificity and sensitivity of previously available techniques (19–22) were insufficient to separate and quantify the single components of silymarin in biological fluids.

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