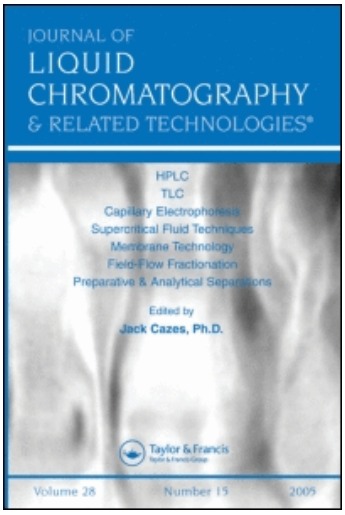

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LIQUID CHROMATOGRAPHIC ASSAY OF SILYBIN IN HUMAN PLASMA AND URINE

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ABSTRACT

A specific and sensitive high-performance liquid chromatographic method for the quantitative determination of silybin and its conjugates in human plasma and urine was developed. Silybin glucuronides and sulfates were calculated as silybin after enzymatic hydrolysis with ß-glucuronidase/arylsulfatase. Extraction from the biological fluid was performed at pH 4 on normal-phase solid extraction columns using tert-butylmethylether as eluent. (+)-Catechin was added as internal standard after the extraction procedure. A normal-phase column was used for the HPLC analysis. The mobile phase consisted of n-hexane/ethanol, acidified with 85% phosphoric acid. Detection was performed by a variable-wavelenght detector at 214 nm. Detection limits of 5 and 25 ng/ml were respectively achieved for free and total (free and conjugated) silybin in plasma, and 100 ng/ml in urine. The method is suitable for pharmacokinetic studies after oral administration of IdB 1016, a lipophilic silybin-phosphatidylcholine complex.

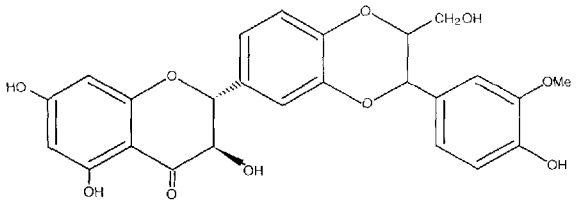

FIGURE 1 Silybin structure.

INTRODUCTION

Silybin is the most important flavanolignan constituent of silymarin, a standardized extract from Silybum marianum, widely used as an antidote in various liver intoxications and endowed with an interesting anti-lipoperoxidant activity (1,2).

The therapeutical application of silybin is limited by its very low bioavailability (3,4,5). In an attempt to improve this property, IdB 1016, a complex of silybin with phosphatidylcholine (6,7), has been prepared. The study of the pharmacokinetic behaviour of silybin orally administered as IdB 1016 in man, required the development of a sensitive and specific assay in biological fluids. In the literature an analytical method for the assay of flavanolignan components of silymarin in biological fluids is described (8,9,10), based on thin-layer chromatography photodensitometry with fluorimetric detection.

This paper deals with the development of an high-performance liquid chromatographic method for the quantitative determination of silybin in human plasma and urine.

Flavonoids are extensively conjugated, mainly in the liver (11), therefore the evaluation of the extent of conjugation of the

drug is of major importance for its pharmacokinetic characterization. In order to allow the quantification of total silybin, that is after deconjugation of glucuronides and/or sulfates, the conditions of an enzymatic hydrolysis using ß-glucuronidase/arylsulfatase are also reported.

MATERIALS AND METHODS

Chemicals

Silybin (m.p. 177-179°C by DSC; $[\alpha]_D^{20}$ = 12.5, C=1, methanol) was isolated from silymarin by column chromatography and purified by crystallization from methanol. IdB 1016 was obtained by complexation of silybin with phosphatidylcholine (6) and characterized by spectroscopic analysis (7). (+)-Catechin was obtained from Fluka (Buchs, CH). ß-Glucuronidase/arylsulfatase (Helix pomatia) was purchased from Boehringer Mannheim (Mannheim, FRG). n-Hexane, methanol, ethanol and tert-butylmethylether eluents (HPLC grade), acetate and citrate buffers, Extrelut 3 columns were supplied by Merck (Darmstadt, FRG).

Apparatus

The liquid chromatograph consisted of a Model 590 HPLC pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model L-4200 Hitachi variable-wavelength absorbance UV-VIS detector (Merck, Darmstadt, FRG).

HPLC Conditions

A LiChrosorb Diol column (150 mm x 3 mm; Merck, Darmstadt, FRG), 5 μm particle size was used. The mobile phase consisted of n-hexane/ethanol (70:30, v/v), acidified with 120 μl/l of 85% phosphoric acid. The flow rate was 0.8 ml/min and the detection was performed at 214 nm.

Extraction Procedure

Plasma

Free silybin. Plasma aliquots (500 μl) were added with 2.5 ml of pH 4 citrate buffer; the samples were then loaded on Extrelut 3 columns and eluted twice with 10 ml of tert-butylmethylether. The eluates were evaporated under nitrogen at 37°C. The residues were added with 10 μl of a (+)-catechin methanolic solution (internal standard, 10 μg/ml) and resuspended in 150 μl of mobile phase. Aliquots (20 μl) were injected for HPLC analysis.

Total (free and conjugated) silybin. To 500 μl of plasma, 1.5 ml of pH 5 acetate buffer and 80 μl of ß-glucuronidase/arylsulfatase (about 0.44 U and 0.21 U of glucuronidase and arylsulfatase activity respectively) were added. After incubation at 37°C for 48 hours, samples were extracted and analyzed as above.

Urine

Urine samples were analyzed only for their total silybin content, that is after enzymatic hydrolysis with ß-glucuronidase/arylsulfatase.

Hydrolysis, extraction and analysis were performed on 500 μl urine as described for plasma.

Calibration Samples

Calibration samples were prepared by adding known increasing amounts of silybin (solution in methanol) to pooled plasma or urine and were processed as described above. The plasma calibration concentrations ranged from 10 to 400 ng/ml for the free silybin determination and from 0.1 to 1.6 μg/ml for the total silybin determination. The urine calibration concentrations ranged from 0.5 to 16 μg/ml.

RESULTS AND DISCUSSION

Plasma

Free silybin. Extraction recovery was determined by comparing peak-height ratio (R) of silybin to internal standard in calibration samples with those obtained by direct injection of methanolic standard solution. Mean recoveries ranged between 90 and 95 percent (Table 1).

The relationship between R values and silybin concentrations, determined by least-squares linear regression, showed to be linear over the concentration range 10-400 ng/ml according to the equation: $y=-0.019+0.006x$ ($r=0.999$). Intra-assay precision and accuracy, assessed at six different concentrations, are showed in Table 2. The detection limit was 5 ng/ml.

Total silybin. The calibration curve of silybin in plasma added with ß-glucuronidase/arylsulfatase showed to be linear over

TABLE 1

Recovery of Silybin from Plasma

Concentration (ng/mL)	Recovery (%) (Mean)	CV (n=3)
10	97.39	4.19
25	92.70	8.42
50	94.39	3.78
100	93.33	2.89
200	90.58	6.18
400	96.16	0.49

TABLE 2

Precision and Accuracy of Silybin Assay in Human Plasma

Actual Concentration μ (ng/mL)	Measured Concentration $\bar{X}$ (n=3) (ng/mL)	Precision CV	Accuracy $\frac{\bar{X}-\mu}{\mu}.100$
10	10.846	6.80	+ 8.46
25	28.353	7.50	+13.41
50	51.928	3.76	+ 3.86
100	100.850	2.79	+ 0.85
200	187.417	6.07	- 6.29
400	405.607	0.49	+ 1.40

TABLE 3

Precision and Accuracy of Silybin Assay in Plasma added with ß-Glucuronidase/Arylsulfatase

Actual Concentration μ (μg/mL)	Measured Concentration $\bar{X}$ (n=3) (μg/mL)	Precision CV	Accuracy $\frac{\bar{X}-\mu}{\mu}.100$
0.1	0.087	8.05	-13.00
0.2	0.191	9.95	- 4.50
0.4	0.402	3.73	+ 0.50
0.8	0.837	9.20	+ 4.63
1.6	1.583	3.92	- 1.06

the range 0.1-1.6 ug/ml according to the equation: y=0.296+5.005x (r=0.999). Intra-assay precision and accuracy, assessed at five concentrations, are reported in Table 3. The detection limit was 25 ng/ml.

In order to determine the enzymatic hydrolysis time, a pooled plasma sample from a volunteer orally treated with IdB 1016 capsules, was incubated for different times in presence of ß-glucuronidase/arylsulfatase. Hydrolysis resulted complete after 20 hours (Figure 2), but after this time a decrease of silybin values was observed. For this reason the stability of silybin in calibration samples under hydrolytic conditions was also verified. Results are reported in Table 4.

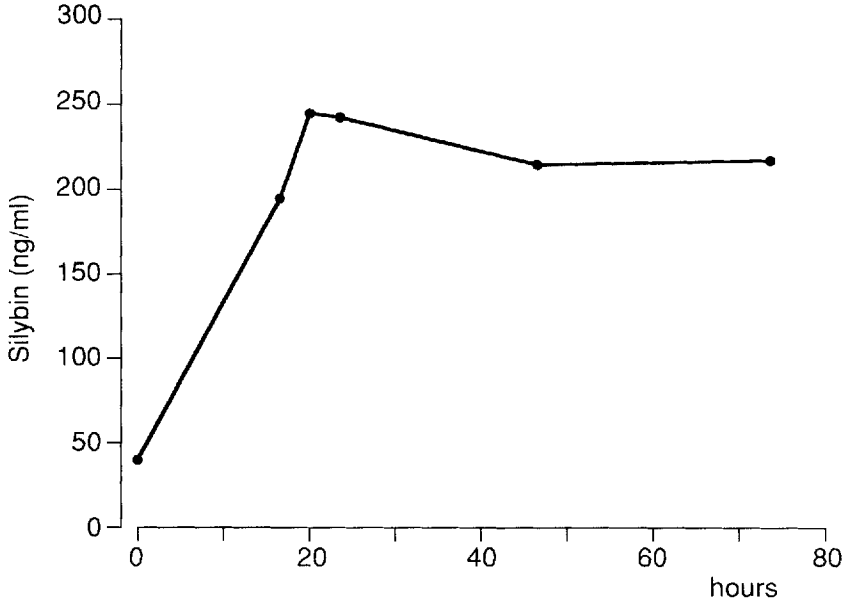

FIGURE 2 Assay of total silybin in human plasma of a volunteer orally treated with IdB 1016 (120 mg as silybin) after incubation with ß-glucuronidase/arylsulfatase at 37°C for different times.

TABLE 4

Stability of Silybin in Blank Plasma after ß-Glucuronidase/Arylsulfatase Treatment

Silybin	Starting Time (t=0)	After Incubation at 37°C (t=48hrs)	
Concentration (μg/mL)	$\bar{R}$ (mean+S.D.)(n=3)	$\bar{R}$ (mean+S.D.)(n=3)	Degradation (%)
0.1	0.732 ± 0.037	0.541 ± 0.061	26.09
0.4	2.307 ± 0.075	1.671 ± 0.335	27.57
1.6	8.218 ± 0.311	6.308 ± 0.664	23.24

R = silybin to internal standard peak height ratio.

TABLE 5

Precision and Accuracy of Silybin Assay in Urine added with ß-Glucuronidase/Arylsulfatase

Actual Concentration μ (μg/mL)	Measured Concentration $\bar{X}$ (n=3) (μg/mL)	Precision CV	Accuracy $\frac{\bar{X}-\mu}{\mu}.100$
0.5	0.312	49.68	-37.60
1.0	0.856	18.46	-14.40
2.0	1.916	16.44	- 4.20
4.0	3.743	1.12	- 6.43
8.0	9.167	14.93	+14.59
16.0	15.506	2.29	- 3.09

The extent of degradation of silybin in calibration samples, incubated at 37°C for 48 hours, in comparison with the basal ones, amounted to about 25%. For this reason calibration curves were processed through the same incubation conditions utilized for analysis samples.

The extent of conjugation could be obtained indirectly by difference between total (after hydrolysis) and free (before hydrolysis) silybin.

Urine

The calibration curve of silybin in urine added with ß-glucuronidase/arylsulfatase showed to be linear over the range 0.5-

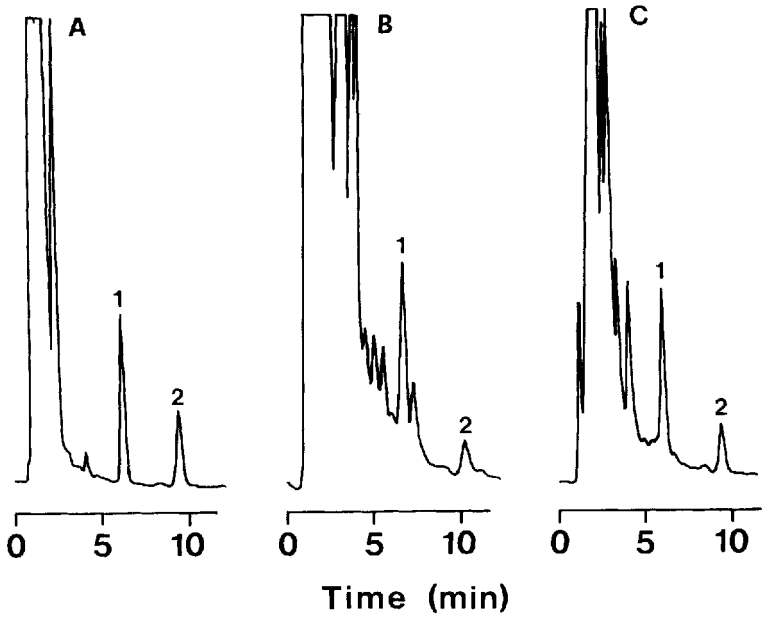

FIGURE 3 Representative chromatograms of:
- Plasma sample obtained from an healthy volunteer 2 hours after oral administration of IdB 1016 (120 mg as silybin); (A) before and (B) after enzymatic hydrolysis.
- Urine sample from the same volunteer obtained within 12 hours after the administration (C).

Peak 1: silybin. Peak 2: internal standard (catechin).

16.0 g/ml according to the equation: y=0.180+0.655x (r=0.996). Intra-assay precision and accuracy, assessed at six concentrations are shown in Table 5. The detection limit was 100 ng/ml.

The present method was used to evaluate the concentrations of silybin in plasma and urine samples from volunteers orally treated with IdB 1016 capsules (120 mg as silybin). Representative chromatograms are reported in Figure 3. The Figure 4 shows a

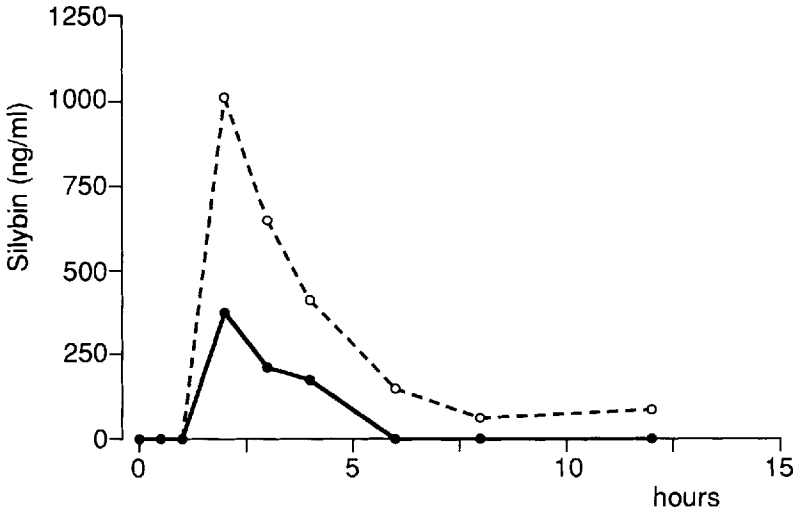

FIGURE 4 Plasma silybin concentration-time profile in an healthy volunteer treated with IdB 1016 (120 mg as silybin). (●) Free silybin. (o) Total silybin (after enzymatic hydrolysis with ß-glucuronidase/arylsulfatase).

typical plasma concentration-time profile for silybin before (free) and after (free and conjugated) enzymatic hydrolysis.

In conclusion the analytical method described in the present paper provides a simple, specific and sensitive HPLC assay for the quantitative determination of silybin and indirectly of its conjugates in human plasma and urine.

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