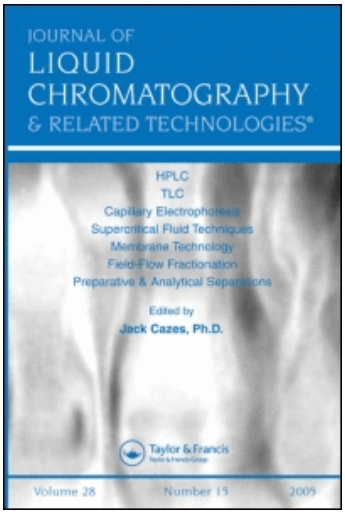

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Hermann Mascher[a]; Christian Kikuta[a]; Roland Weyhenmeyer
[a] Pharm-Analyt Laboratory GmbH, Traiskirchen, Austria

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DIASTEREOMERIC SEPARATION OF FREE AND CONJUGATED SILIBININ IN PLASMA BY REVERSED PHASE HPLC AFTER SPECIFIC EXTRACTION

HERMANN MASCHER*[1], CHRISTIAN KIKUTA[1], AND ROLAND WEYHENMEYER[2]

[1]*Pharm-Analyt Laboratory GmbH*
Wiener Strasse 37
A-2514 Traiskirchen, Austria
[2]*Madaus AG*
D-W-5000 Cologne 91, Germany

ABSTRACT

The analytical method outlined herein makes it possible, for the first time ever, to detect the diastereomers of silibinin separately in human plasma following oral administration of silymarin or silibinin to human subjects in the course of pharmacokinetic investigations, with unprecedentedly low detection limits. The method permits detection of both free (= unconjugated) silibinin diastereomers and of silibinin dia-stereomers following enzymatic cleavage of the silibinin glucuronides and sulphates. The detection limit per diastereomer is 2.5 ng/ml for the free silibinin and 5 ng/ml following enzymatic cleavage. A crucial aspect of this method is its extremely selective extraction and re-extraction of the silibinin, with recovery rates of around 80 %. The diastereomers are separated, without derivatization, on a reversed phase C18-column followed by UV detection at 285 nm. The linearity in the range tested (6 - 98 ng for each diastereomer / ml plasma in the case of free silibinin and 7- 1829 ng for each diastereomer / ml plasma in the case of total silibinin) is very good indeed. The day to day variation (3 days, 3 concentrations; each n = 12) is lower than 4.8% (CV) with an accuracy of -1.1% to 6.1%.

*) To whom correspondence should be adressed

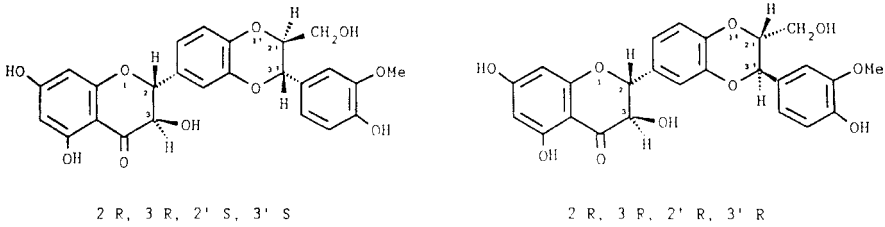

FIGURE 1: Chemical structure of silibinin diastereomers

INTRODUCTION

Silymarin is the antihepatotoxic principle of the milk thistle (Silybum marianum). It consists of the different isomers silidianin, silicristin, isosilibinin and silibinin (1). Silibinin is the principal component and the main active substance. Silidianin is very heavily metabolized (2), while silicristin is only absorbed to a very slight extent in the gastrointestinal tract (2). Virtually no material has been published on the detection of silibinin from body fluids in connection with clinical and pharmacokinetic studies on human subjects. The few papers there are that deal with the determination of silibinin in biological fluids (3,4,5,6) are based upon two different analytical methods, only one of which - the TLC method by Lorenz (4) - has actually been published and is therefore possible to replicate. In this latter method, silibinin and isosilibinin are determined as a summation peak after cleavage of the glucuronides and sulphates. The detection limit from plasma was 50 ng total silibinin + isosilibinin/ ml. The 2nd method, by Barzaghi (6), is based on HPLC and appears to be able to separate silibinin and isosilibinin. As the detection limit appears to be somewhat lower (5 ng/ml for free silibinin and 25 ng/ml for total silibinin), this method has proved capable of detecting both unconjugated silibinin and conjugated silibinin (silibinin glucuronides and sulphates). Unfortunately, however, since this latter publication deals with the pharmacokinetics of silibinin, it gives no analytical details of the method itself. The investigations carried out by Quercia (1) clearly demonstrate not only that silymarin contains 4 isomers, but also that silibinin and isosilibinin each consist of diastereomers.

The twinfold objective of the present paper was, firstly, to obtain considerably lower detection limits for silibinin in plasma, in order to determine the unconjugated silibinin and the total (conjugated + unconjugated) silibinin after enzymatic cleavage for a sufficient period of time following the administration of the medicament, and secondly, to achieve separation of the diastereomers (Fig. 1) in order to gain new insights into both the metabolism and the pharmacokinetics of silibinin and its effects on the pharmacodynamics.

EXPERIMENTAL

Apparatus and chemicals

The HPLC equipment comprised a HP-1090 M liquid chromatograph (Hewlett-Packard, USA) with a Spectromonitor 3100 variable-wavelength UV detector (Milton Roy, USA) and a PE-Nelson 2600 data system (Perkin Elmer, USA).

The solvents and reagents used were of HPLC grade from Rathburn (Scotland) or of analytical grade from E.Merck (Germany). The glucuronidase/arylsulphatase enzyme solution made from Helix Pomatia was from Boehringer-Mannheim (No. 127698), FRG, and was purified in our laboratory using C 18 solid phase extraction columns [1 ml enzyme solution twice through a preconditioned C18 precolumn filled with 100 mg material (Analytichem, USA)].

The analysis column was filled with Nucleosil 120 3C18 (80x4 mm id, SRD-Pannosch, Vienna, Austria). The pure silibinin substance was from Madaus AG (Cologne, Germany).

HPLC

The mobile phase consisted of 50 % methanol and 50 % 0.02 M perchloric acid. The flowrate was 1.0 ml/min. The column temperature was kept at room temperature. Detection took place in UV at 285 nm.

A) Free silibinin in plasma

Sample preparation

After the plasma samples had been thawed, 0.2 ml of 0.1 M Na_2HPO_4 solution and

4 ml of diethylether were added to 1 ml of plasma, which was then shaken vigorously by hand for 1 min. Following centrifugation at >1000 g, the aqueous phase was frozen out in acetone dry ice and the organic phase was quantitatively decanted into a tapered centrifuging tube. After 0.75 ml of 0.1 M Na_2CO_3 solution had been added, this was shaken for 1 min by hand and then centrifuged at >1000 g. 0.5 ml of the aqueous phase was transferred into an autosampler vial by means of a Hamilton syringe, and then mixed with 0.15 ml of 1M H_3PO_4. 100 μl of this mixture was then injected.

Validation

The method was validated by adding various different quantities of silibinin to pooled human plasma. The resulting concentrations were 12.2, 24.4, 48.8, 97.5 and 195.1 ng of silibinin (diastereomer mixture) per ml of pool-plasma. These calibration series were used to test both the linearity of the method and its precision, accuracy and absolute recovery rate by comparison with non-extracted aqueous standard solutions.

B) Total silibinin in plasma

Sample preparation

After the plasma samples had been thawed, 1 ml of pH 5 buffer solution (acetic acid/acetate) and 50 μl of purified enzyme solution (approx. 5000 Fishman-U and 40000 Roy-U) were added to 1 ml of plasma, which was then warmed for 4 h at 37°C in order to bring about cleavage of the glucuronides and sulphates of the silibinin. Once the solution had cooled, 0.5 ml of carbonate buffer solution ($NaHCO_3/Na_2CO_3$) was added, in order to achieve a pH value of 8.5. After 4 ml of diethylether had been added, this was shaken vigorously for 1 min by hand and then centrifuged at >1000 g. The aqueous phase was then frozen out in acetone dry ice and the ethereal phase was treated in the same way as described for free silibinin above.

Validation

The method was validated by adding various different quantities of silibinin to pooled human plasma. The resulting concentrations were 14.3, 28.6, 57.1, 114.3, 228.6, 457.2, 914.4, 1828.8 and 3657.5 ng of silibinin (diastereomer mixture) per ml of pool-

plasma. These calibration series were subjected to the entire analytical procedure (including enzyme addition), so as to test the linearity, precision and accuracy of the method.

RESULTS AND DISCUSSION

Separation

Our investigations indicate that chromatographic separation of the diastereomers as described in Quercia (1) may best be performed on reversed phase C 18 systems. Chains shorter than this, e.g. C 8 or C 4, result in distinctly inferior separation, as does phenyl. Acetonitrile also results in greatly inferior separation by comparison with methanol. Fig. 2 shows a plasma calibration sample and test-subject samples following administration of silymarin or silibinin.

Selectivity

We have been working on the detection of silibinin, with and without diastereomeric separation, over a considerable period of time. Successful detection of free silibinin from plasma was achieved very early on, using column switching methods and/or concentration enrichment on C 18 pre-columns on line. However, there were serious problems with determination following enzymatic cleavage, as various commercially available enzyme solutions came with numerous disturbing impurities right from the outset, and as substances resulting from cleavage prevented determination with pre-column purification. Not even electrochemical detection - which normally permits highly sensitive detection of silibinin - provided a solution here. It was only the specific extraction and re-extraction method outlined above which made it possible to achieve selective detection with low detection limits and diastereomeric separation. Without diastereomeric separation, for example, we achieved very sensitive detection on diol and cyano phases a considerable time ago (unpublished results).

One of the crucial points in this method - as mentioned above - is the fact that the pH value is set at pH 8 - 8.5 prior to extraction, with reextraction being performed at around pH 10.5 - 11. As the pk value of the silibinin (phenolic group) is around 9.5, the bulk of the silibinin is undissociated at pH 8 - 8.5 and - due to its lipophilic properties - may be extracted into ether.

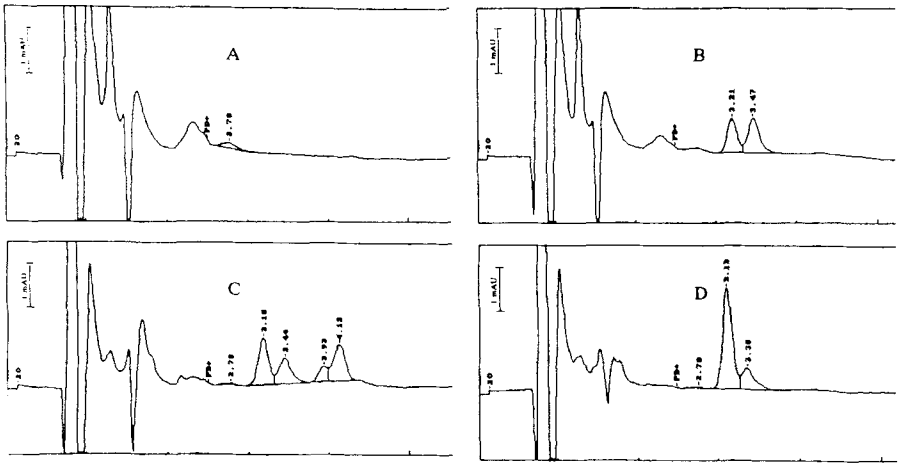

FIGURE 2: Determination of free silibinin in human plasma
A) Pool-plasma without silibinin
B) Pool-plasma spiked with 93.2 ng silibinin /ml
C) Subject plasma after oral administration of Silymarin (Legalon®)
103.2 ng silibinin (as diastereomer 1 + 2)/ ml plasma
Peaks at 3.93 and 4.12 min: isosilibinin (diastereomers)
D) Subject plasma after oral administration of silibinin
157.3 ng silibinin (as diastereomer 1 + 2)/ ml plasma
Retention time: approx. 3,1 min silibinin diastereomer 1
approx. 3,4 min silibinin diastereomer 2
HPLC conditions: mobile phase: methanol/0.02M perchloric acid (50:50 v/v); column: Nucleosil 120 3C18, 80x4 mm id, detection: UV at 285 nm, 0.005 AUFS.

At this pH value, organic acids are no longer co-extracted. Reextraction is from ether, with a buffer solution, at around pH 10.5 - 11. At this pH value, all neutral lipophilic molecules remain in the ether, as do the co-extracted bases. In this way, only molecules with phenolic groups are re-extracted. These two extractive steps constitute an extremely selective pre-purification procedure which also ensures adequate recovery rates over the entire working range (Tab. 1). The freezing out of the aqueous phase permits quantitative and reproducible decanting of the diethylether. Therefore it is possible to dispense with an internal standard (a suitable internal standard that does not already occur naturally is in any case virtually impossible to find).

TABLE 1

Free Silibinin in Plasma

Conc. spiked ng / ml	n	Precision ± CV %	Accuracy %	Recovery abs.%±CV%
12.2	3	5.6	1.7	73.2 ± 4.26
24.4	3	2.0	1.7	81.0 ± 1.63
48.8	3	1.7	- 4.8	75.8 ± 1.31
97.5	3	4.3	0.5	80.3 ± 3.44
195.1	3	1.8	0.1	80.0 ± 1.44

A) Free silibinin in plasma

Linearity

In the calibration series, the linear regression between the spiked plasma concentrations and the peak area (sum of diastereomers 1 and 2) was determined after analysis of the calibration samples. When multiple analysis was carried out (with n = 24 plasma samples) over the calibration range from 12 - 195 ng/ml, a typical value of 205.98 was obtained for the slope, with an intercept of 129.6 and a correlation coefficient of 0.9994. The detection limit was calculated as 5 ng for both diastereomers (S/N: 2/1), which is 2,5 ng of each silibinin diastereomer / ml plasma.

Variation

Both the small coefficients of variation (1,7 - 5,6% CV; 12 - 195 ng / ml pool-plasma), the high accuracy achieved and the recovery may be seen from Table 1.

Pharmacokinetics

A representative test subject was selected from a randomized cross-over study involving the administration of oral silymarin (Legalon ®, Madaus AG, FRG), in order to show the concentrations of silibinin diastereomers (unconjugated silibinin in plasma)

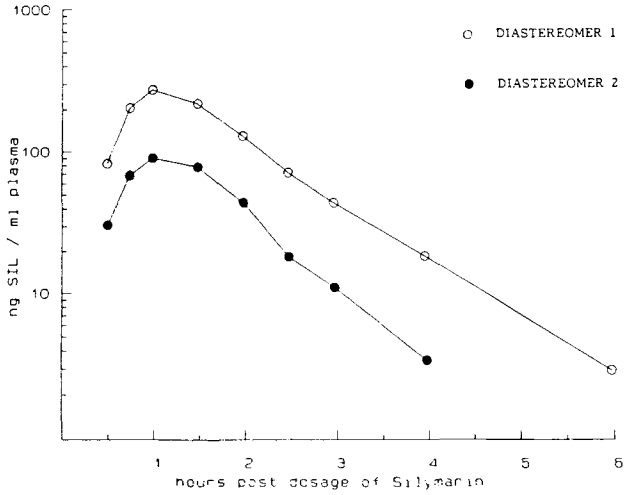

FIGURE 3: Concentration of free silibinin (diastereomer 1 + 2) in human plasma from one selected volunteer after oral administration of 100mg silibinin in ther form of Silymarin (Legalon®).

over a certain period of time (7). The dosage corresponded to 100 mg of silibinin (Fig. 3).

B) Total silibinin in plasma

Linearity

In the calibration series, the linear regression between the spiked plasma concentrations and the peak area (sum of diastereomers 1 and 2) was determined after analysis of the calibration samples. When multiple analysis was carried out over the range from 14 - 3658 ng/ml (with n = 26 plasma samples) a typical value of 166.08 was obtained for the slope, with an intercept of -386.8 and a correlation coefficient of 0.9997. The slopes of the calibration lines over several days were very constant (4 days; slope 164.59 +/- 1.30 %) . The detection limit was calculated as 10 ng for both diastereomers (S/N : 2/1), which is 5 ng of each silibinin diastereomer / ml plasma).

TABLE 2

PRECISION and ACCURACY: Total Silibinin in Plasma after Enzymatic Cleavage

Conc. found ng / ml	Mean ng / ml	Precision ± CV %	Conc. spiked ng / ml	Accuracy %
14.2	15.4	13.5	14.3	7.9
17.8				
14.2				
28.1	28.7	1.9	28.6	- 0.4
28.8				
29.2				
51.9	57.1	10.4	57.1	0.0
63.6				
55.9				
104.5	112.4	9.3	114.3	-1.7
124.2				
108.5				
206.5	217.4	4.6	228.6	- 4.9
219.8				
226.0				
437.4	446.5	1.8	457.2	- 2.3
452.2				
449.9				
882.9	902.2	3.3	914.4	- 1.3
887.3				
936.4				
1809.4	1861.4	2.8	1828.8	1.8
1913.0				
1861.9				
3604.3	3659.6	1.6	3657.5	0.1
3718.5				
3656.0				

TABLE 3

DAY TO DAY VARIATION WITH SPIKED QUALITY CONTROL SAMPLES
TOTAL SILIBININ (3 different days, each day n = 4)

Conc.spiked ng / ml	Conc.found day 1 mean	Conc.found day 2 mean	Conc.found day 3 mean	Mean ng / ml	CV (±%)	Accuracy
56.0	55.6	53.6	57.0	55.4	3.1	- 1.1
374.6	382.2	359.3	395.6	379.0	4.8	1.2
1851.6	2007.1	1924.4	1963.2	1964.9	2.1	6.1

Variation

Both the coefficients of variation (1,6 - 13,5 % CV; 14 - 3658 ng/ml pool-plasma) and the accuracy may be seen from Table 2 (primary calibration).
Day to day variation may be seen from Table 3 in the concentration range between 56 and 1852 ng/ml pool-plasma.The variation is lower than 4.8% (CV) and the accuracy is between -1.1% and 6.1%.

Pharmacokinetics

The results for the same test subject as with "Free silibinin" were selected, i.e. from the same cross-over study (7), and represented in Fig. 4. In the case of Figs. 3 and 4, it must be borne in mind that the preparation administered had a diastereomer ratio of approx. 0.6 : 1. The distinctly varying glucuronisation / sulphatisation of the diastereo-mers is to be the subject of a further planned publication. In order to enable comparison with the results in the publication by Barzaghi (6), the concentrations of both diastereo-mers were added together (Fig. 5). Following administration of silymarin (Legalon R), we obtained similar concentrations of free and total silibinin to those obtained by Barzaghi (6) following administration of a silibinin phosphatidyl choline complex. Barzaghi, however, obtained distinctly lower silibinin concentrations following adminis-tration of silymarin (6), suggesting insufficient bioavailability of the galenic

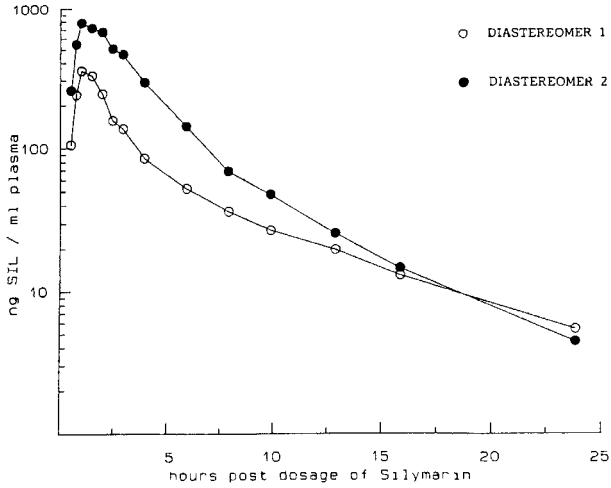

FIGURE 4: Concentration of total silibinin (diastereomer 1 and 2) in human plasma from one selected volunteer after oral administration of 100mg silibinin in the form of Silymarin (Legalon®).

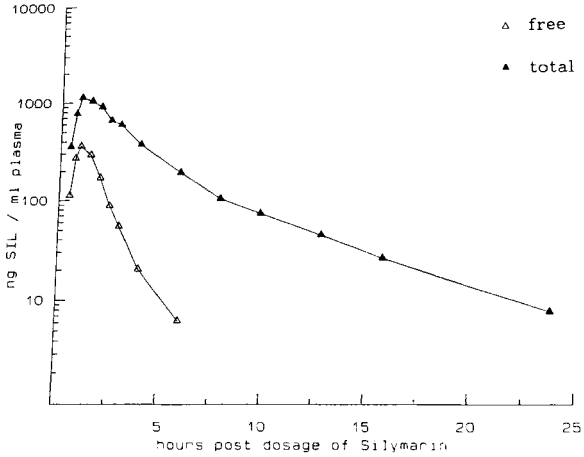

FIGURE 5: Concentration of free and total silibinin (sum of diastereomer 1 and 2) in human plasma from one selected volunteer after oral administration of 100mg silibinin in the form of Silymarin (Legalon®).

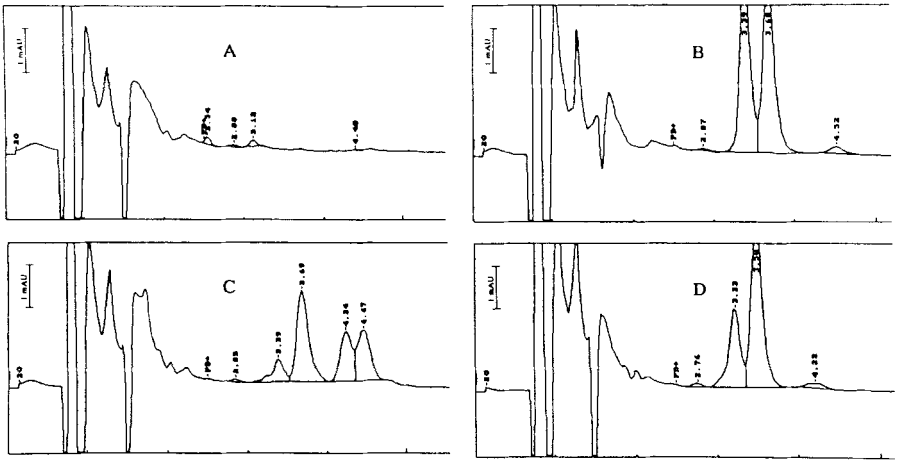

FIGURE 6: Determination of total silibinin in human plasma after enzymatic cleavage
A) Pool-plasma without silibinin
B) Pool-plasma spiked with 369 ng silibinin /ml
C) Subject plasma after oral administration of Silymarin (Legalon®)
140.2 ng silibinin (as diastereomer 1 + 2)/ ml plasma
Peaks at 4.24 and 4.47 min: isosilibinin (diastereomers)
D) Subject plasma after oral administration of silibinin
277.7 ng silibinin (as diastereomer 1 + 2)/ ml plasma
Retention time: approx. 3.3 min silibinin diastereomer 1
approx. 3.6 min silibinin diastereomer 2
HPLC conditions: as described in Fig. 2

test-subject samples following administration of silymarin and silibinin.

This method was used to obtain initial results on the diastereomer ratio of isosilibinin (1) following the administration of silymarin (Legalon R). These results are not the subject of the present publication, however.

CONCLUSION

To sum up, it can be said that the analytical method described herein makes it possible, for the first time ever, to detect both silibinin diastereomers following the administration of silymarin or of silibinin as a monosubstance. Owing to its excellent

detection limits, this method is very suitable indeed for basic research into the pharmacokinetics and pharmacodynamics of silymarin and/or silibinin. Recent results obtained following the administration of various galenical formulations of silibinin as a monosubstance show definite differences in the ratio of the diastereomers as compared to the results of the administration of silymarin described in this paper.

Acknowledgement

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