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Journal of Chromatography B, 670 (1995) 267–277

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Two high-performance liquid chromatographic assays for the determination of free and total silibinin diastereomers in plasma using column switching with electrochemical detection and reversed-phase chromatography with ultraviolet detection

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First received 1 February 1995; revised manuscript received 22 March 1995; accepted 10 April 1995

Abstract

A combination of two stereoselective assays was developed using column-switching HPLC with electrochemical detection for the determination of free (unconjugated) silibinin and RP-HPLC with UV detection for the measurement of total (free and conjugated) silibinin in human plasma. After extraction of free silibinin and the internal standard hesperetin with diethyl ether the compounds were pre-separated on a RP-CN column. A cut fraction of eluate containing the analytes was then transferred to the RP-18 main column by means of a switching valve for final separation of the compounds. The limit of quantification with electrochemical detection for free silibinin was 0.25 ng/ml per diastereomer. For the determination of total silibinin diastereomers all conjugates were cleaved enzymatically using β-glucuronidase/arylsulfatase at pH 5.6 followed by extraction with diethyl ether of the pH 8.5 alkalized solution. Separation of the diastereomers and of the internal standard naringenin was achieved on a RP-18 column. The limit of quantification with UV detection at 288 nm for total silibinin was 5 ng/ml per diastereomer. Both assays were successfully applied to the stereospecific analysis of silibinin in plasma samples from a pharmacokinetic study of silymarin in human volunteers.

1. Introduction

Silibinin is the principal component and main active substance of silymarin, a standardized extract of flavanolignans from the fruit of *Silybum marianum* (L.) Gaertner which is widely used as an antihepatotoxic drug. In plants only two from the theoretically possible sixteen diastereomers of silibinin are produced (Fig. 1). The absolute assignment was achieved by X-ray analysis [1] of the natural diastereomeric mixture showing that isomerism occurs only in the benzodioxane part of the molecule. Up to now the individual diastereomers cannot be separated on a preparative scale. Silibinin concentrations in body fluids have been determined using either thin-layer chromatography (TLC) with fluorometric detection [2] or high-performance liquid chromatography (HPLC) with UV detection [3,4]. Techniques employing TLC lack both sensitivity and specificity thus limiting their use.

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0378-4347/95/$09.50
SSDI 0378-4347(95)00168-9

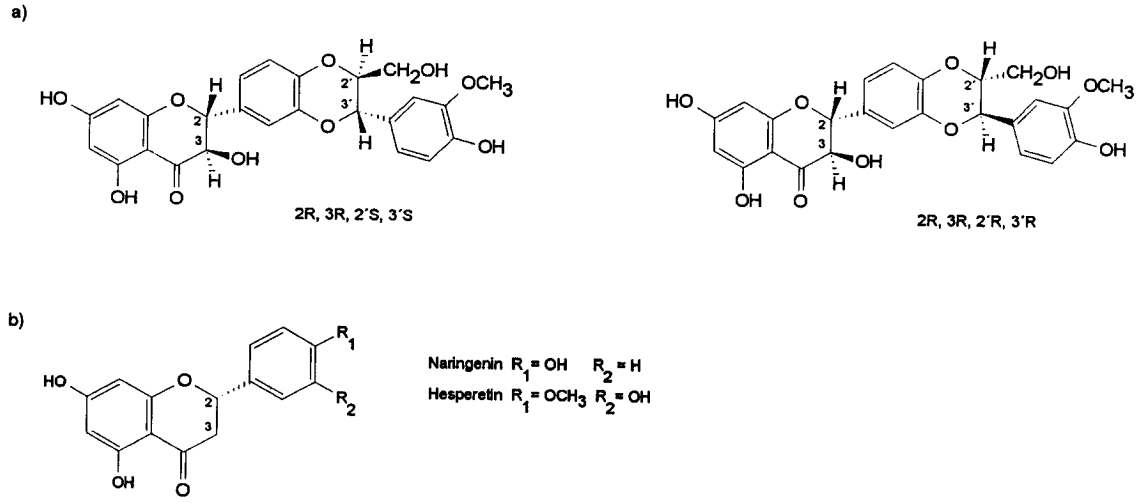

Fig. 1. Structures of silibinin diastereomers (a) and of the internal standards naringenin and hesperetin (b).

Previous HPLC assays are either not stereoselective [3] or not sensitive enough [4] to monitor free silibinin plasma concentrations during the necessary sampling period of 24 h after single oral doses. Since silibinin is metabolized exclusively to glucuronate and sulfate conjugates which are partly hydrolysed and cycled enterohepatically, not only parent drug but also total silibinin including the metabolites should be measured in plasma.

Silibinin may act as a radical scavenger [5]. We, therefore, tried electrochemical detection for the determination of silibinin in plasma. By combining this detection method with column-switching we achieved a 10-fold increase in sensitivity to 0.25 ng/ml per diastereomer. For the analysis of total silibinin (including conjugated metabolites) electrochemical detection could not be used because of interfering material resulting from enzymatic hydrolysis. Nevertheless, we were able to enhance sensitivity to 5 ng/ml per total diastereomer and to simplify the extraction procedure. Internal standardization improved the robustness of both assays. The applicability of the methods is illustrated for the analysis of human plasma samples from a study in 10 healthy volunteers where the pharmacokinetics of total and free diastereomers were evaluated.

2. Experimental

2.1. Chemicals

Silibinin (certified reference compound) was provided by MADAUS AG (Köln, Germany). Hesperetin and naringenin were purchased in CHR quality from C. Roth (Karlsruhe, Germany). Glacial acetic acid, diethyl ether, boric acid, sodium acetate and sodium dihydrogen phosphate monohydrate were supplied in analytical quality by Merck (Darmstadt, Germany). Acetonitrile and methanol both LiChrosolv grade were also obtained from Merck. HPLC-grade water was generated by double quartz glass distillation. β-Glucuronidase/arylsulfatase crude solution from Helix pomatia was purchased from Boehringer (Mannheim, Germany) and purified by passing it twice through preconditioned (methanol, water) Chromabond C_{18} SPE columns (Macherey-Nagel, Düren, Germany). Boric acid buffer solution pH 8.5 (0.5 *M*)

was prepared by dissolving 3.1 g of boric acid in approximately 70 ml of water, adjusting the pH to 8.5 with 1 *M* sodium hydroxide solution and filling up the volume to 100 ml with water.

2.2. Apparatus

HPLC with column-switching

The chromatographic system consisted of two L-6200 intelligent pumps, an AS-2000 auto sampler, an L-5025 column thermostat (all from Merck-Hitachi, Darmstadt, Germany), an ELV 7000 high speed actuator equipped with a Rheodyne 7000 switching valve (W. Krannich, Göttingen, Germany) and an ESA Coulochem II electrochemical detector Model 5200 in combination with a Model 5011 analytical cell (ESA, Bedford, MA, USA). The signals of the detector were analyzed using a PE Nelson 900 series interface with Turbochrom III (version 3.3) software (PE Nelson, Cupertino, CA, USA).

Standard RP-HPLC

The chromatographic system was composed of an L-6200 intelligent pump, an AS-2000 auto sampler, an L-5025 column thermostat (all from Merck-Hitachi) and an LC 95 variable-wavelength UV detector (Perkin Elmer, Norwalk, CT, USA). The signals of the detector were analyzed as described above in HPLC with column-switching.

2.3. Chromatography of free silibinin diastereomers

Separation of the compounds was achieved using two different columns employing the column-switching technique (Fig. 2). A Nucleosil 100-5 CN column (particle size 5 μm, 250 × 4 mm I.D., Macherey-Nagel) was used for pre-separation with a mobile phase consisting of acetonitrile and 0.2 *M* acetic acid (20:80, v/v) delivered by pump A at a flow-rate of 1 ml/min. From 3.6 to 5.6 min, the valve switched the flow path to the analytical column (LiChrospher 100 RP-18, 5 μm particle size, 250 × 4 mm I.D., Merck) transferring the cut fraction of eluate containing the analytes. After the valve had switched to the initial position the final separation of the compounds on the analytical col-

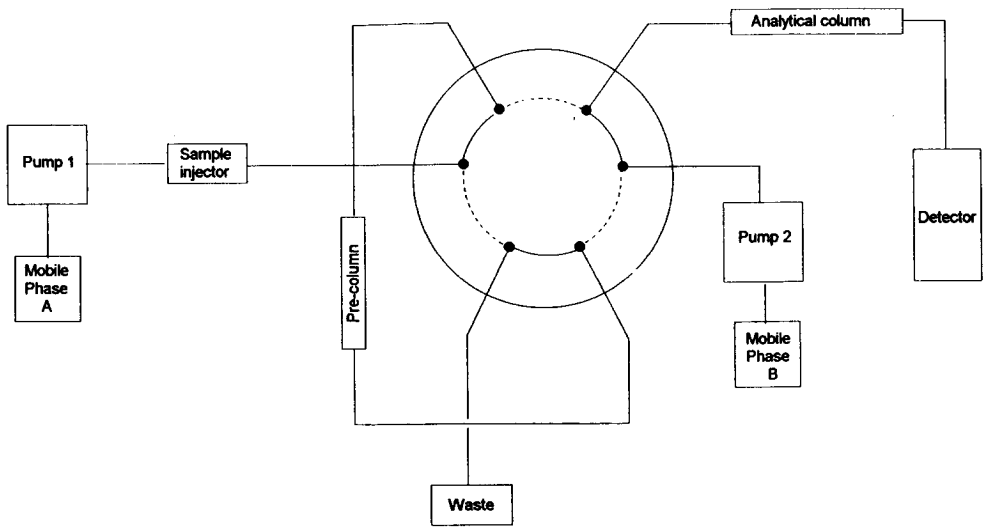

Fig. 2. Schematic diagram of the column-switching system. The plasma extract was injected onto the pre-column and endogenous components from the matrix were washed to waste with mobile phase A delivered by pump 1. By switching the valve a cut fraction of mobile phase A was transferred to the analytical column for final separation of the analytes with mobile phase B delivered by pump 2.

umn was achieved using a mixture of methanol and 0.1 *M* sodium dihydrogen phosphate buffer (pH 3) (46:54, v/v) delivered by pump B at a flow-rate of 1 ml/min. Meanwhile the pre-column was washed with acetonitrile at a flow-rate of 1 ml/min for 6 min and equilibrated for 14 min at initial conditions. The signal of the detector was recorded within an 8-min time interval starting ca. 16 min after the injection resulting in a total run time of 25 min from injection to injection. The electrochemical detector was set at a potential of +300 mV for electrode 1 and +700 mV for electrode 2. The columns were maintained at a temperature of 40 ± 1°C.

2.4. Chromatography of total silibinin diastereomers

Analysis of the compounds was performed using a Nucleosil 120-3 C_{18} column (3 μm particle size, 125 × 3 mm I.D.) coupled to a Nucleosil 120-5 C_{18} guard column (5 μm particle size, 11 × 3 mm I.D., both Macherey-Nagel) with a mobile phase consisting of a 60:40 (v/v) mixture of methanol and 0.1 *M* phosphate buffer pH 3 at a flow-rate of 0.5 ml/min. After the analytes and the internal standard had been eluted, the mobile phase was changed to 100% methanol at 0.5 ml/min for 2 min followed by 21 min of equilibration at initial conditions. The total run time was 45 min. The column was maintained at a temperature of 40 ± 1°C and the UV detector was set at a wavelength of 288 nm. The retention times were 28.8 and 32.2 min for the silibinin diastereomers and 18.8 min for the internal standard.

2.5. Sample preparation

Free silibinin

Silibinin and the internal standard hesperetin were extracted from 1 ml of human plasma by shaking with 5.5 ml diethyl ether for 10 min. After centrifugation at 2000 *g* (12°C) for phase separation the organic phase was transferred into 5-ml micro reaction vessels (Supelco, Bad Homburg v.d.H., Germany) and evaporated at 45°C under a stream of nitrogen. The residue was redissolved in 50 μl of methanol, vortexed for 3 minutes, centrifuged and 40 μl of the supernatant were used for HPLC analysis.

Total silibinin

For enzymatic cleavage 1 ml plasma containing the internal standard naringenin (50 ng) was incubated with 1 ml of 1 *M* sodium acetate buffer pH 5.6 and 30 μl of a purified solution of β-glucuronidase/arylsulfatase from Helix pomatia for 3 h at 37°C and 55 r.p.m. shaking. The enzyme solution was purified by passing twice through preconditioned (methanol, water) Chromabond C_{18} SPE columns. After adding 2 ml of borate buffer solution pH 8.5 (0.5 mol/l) silibinin and the internal standard naringenin were extracted into 5.5 ml diethyl ether by shaking for 20 min. After centrifugation for 10 minutes at 2000 *g* (12°C) the organic phase was transferred into 5-ml micro reaction vessels and evaporated at 45°C under a stream of nitrogen. The residue was redissolved in 30 μl of methanol, vortexed for 1 min, centrifuged and 6 μl of the supernatant were used for HPLC analysis.

2.6. Calibration

Free silibinin

Stock solutions of silibinin and the internal standard hesperetin were prepared by dissolving each compound in methanol to a concentration of 10 mg/100 ml. The working solutions in methanol were obtained by diluting the stock solutions. All standard solutions were kept at 4°C for up to three months.

For calibration 1 ml of human plasma was spiked with 20 μl of the internal standard (10 ng) and 40 μl of the silibinin working solutions in the range of 100, 50, 25, 10, 5, 2.5, 1, 0.5 ng corresponding to a final concentration of 50–0.25 ng/ml of each diastereomer. Quantification was achieved by measuring the peak heights of each silibinin diastereomer and the internal standard. A standard curve of the peak height ratios versus the concentration in human plasma was plotted separately for each diastereomer and the slope and intercept were determined by weighted ($1/y$) inverse linear regression.

Total silibinin

Stock solutions of silibinin and the internal standard naringenin were prepared as described above.

For calibration 1 ml of human plasma was spiked with 50 μl of the internal standard (50 ng) and 50 μl of the silibinin working solutions in the range of 200, 100, 50, 25, 10 ng corresponding to a final concentration of 100–5 ng/ml of each diastereomer. Quantification was achieved as described for free silibinin.

2.7. Method validation (method accuracy and precision)

The reliability of the analytical method was estimated by preparing a series of calibration lines ranging from 100 ng/ml to 0.5 ng/ml for free silibinin and from 200 ng/ml to 10 ng/ml for total silibinin, respectively. The intra-day repeatability of the method was assessed for the determination of free silibinin by repeated analysis of spiked plasma samples at three concentration levels (25, 5 and 0.5 ng/ml, $n = 7$) and at two concentration levels (100 and 12.5 ng/ml, $n = 7$) for total silibinin diastereomers. From these data C.V. (%) (coefficient of variation) and R.E. (%) (relative error between found and nominal value) were derived to characterize the intra-day as well as the inter-day/inter-run precision and accuracy of the assay.

The recovery of each diastereomer was assessed at two concentration levels by comparing the peak heights after extraction with the peak heights obtained from direct injection of equivalent quantities of the pure standards.

2.8. Pharmacokinetic study

As part of a silymarin pharmacokinetic study 10 healthy male volunteers (mean age 57.7 ± 10.0 years, mean weight 79.2 ± 10.9 kg, mean height 172.9 ± 4.5 cm) received 140 mg silymarin, equivalent to 53.2 mg of silibinin, as one single oral dose. Blood samples were collected before the dosage as well as 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 14, 16, 24, 26 h after drug administration. The samples were centrifuged and the plasma removed and stored frozen at −20°C until analysis. The individual areas under the concentration–time curves, $AUC(0 - t_{last})$, were calculated using the linear-trapezoidal rule from 0 h to the last detectable concentration.

3. Results and discussion

3.1. Sample preparation and extraction

The cleavage of silibinin glucuronide and sulfate conjugates for the determination of total silibinin plasma concentrations had to be performed under mild enzymatic conditions as silibinin decomposes if subjected to acid hydrolysis. The crude enzyme solution was purified before use by passing twice through RP-18 cartridges to remove compounds of low molecular mass leading to interfering peaks in the chromatogram. Applying this purification step the former dark brown mixture changes its colour to a clear light brown solution without a substantial loss in enzymatic activity. By adjusting the pH to 8.5 before the extraction it was possible to separate the undissociated phenolic analytes from dissociated organic acids which produced interfering peaks in the chromatogram. Unconjugated silibinin was extracted directly from plasma without adjusting pH to 8.5 as co-extracted organic acids did not affect the chromatography using the column-switching technique. The absolute recovery of the silibinin diastereomers after extraction from plasma was at least 60% in both assays.

3.2. Chromatography and detecting conditions

Total silibinin

The highly sensitive column-switching technique with electrochemical detection could not be applied to the analysis of total silibinin diastereomers because the necessity of an enzymatic hydrolysation step implies the extraction of innumerable electrochemically active compounds which not only reduce the detector's sensitivity but also produce various interfering peaks. Thus, a separate RP-HPLC assay with UV detection

was developed for the determination of total silibinin in plasma. Naringenin, a flavanone compound like silibinin, was used as the internal standard and the detector was set at a wavelength of 288 nm which is the absorption maximum of the silibinin diastereomers. Fig. 3 shows chromatograms of two spiked samples containing 200 and 12.5 ng/ml per diastereomer, a representative sample from the pharmacokinetic study taken 1 h after drug administration and a blank plasma extract. The retention times were 18.3 min for the internal standard naringenin and 28.1 and 31.4 for the two isomers, respectively. There were no interfering peaks detected in blank plasma. After separation of the analytes the column had to be flushed with methanol to remove late-eluting components. The quantification limit of 5 ng per diastereomer was sufficient for monitoring total silibinin plasma levels over a period of 26 h after single oral doses of silymarin.

Free silibinin

Typical chromatograms for free silibinin of blank and spiked plasma extracts (0.25 and 5 ng/ml per diastereomer) as well as of a sample from the silymarin study taken 4 h after drug administration are presented in Fig. 4. The relative retention times were 5.4 min for the internal standard hesperetin and 2.8 and 4.1 min for the two isomers, respectively. Blank plasma was free of interfering peaks. Using column switching for the analysis of free silibinin diastereomers it was necessary to estimate the appropriate time-window for a transfer of the analytes to the main column by monitoring the eluate of the pre-column with UV detection at 288 nm. The required 2 min transfer time resulted in a total transfer volume of 2 ml of mobile phase containing silibinin and the internal standard. While a short column (125 × 4 mm I.D.) was sufficient for a fast pre-separation a longer (250 × 4 mm I.D.) column was needed for the final separation retaining the compounds long enough to provide the time necessary for equilibration after the change from 2 ml acetate-buffered sample to the phosphate-buffered eluent. To avoid a noisy signal of the electrochemical detector both eluents had to be adjusted to the same pH value (pH 3) regardless of differences in molarity or ionic strength. Among many compounds tested hesperetin proved to be the ideal internal standard: due to its similar chemical structure (flavanone, 5,7-diphenol) it eluted shortly after the analytes and it was extracted and oxidized as easily as the silibinin diastereomers. The detector settings chosen (see Section 2.3) were the result of several optimization steps to find out maximum sensitivity combined with sufficient specificity. The pre-potential was set at +300 mV in order to suppress interfering peaks without oxidizing silibinin already and thus decreasing the sensitivity. The potential of the electrode 2 was set at +700 mV which yielded the highest silibinin signal output and kept interfering peaks from the matrix at a minimum. Since the individual diastereomers are not available as pure isolated standards, the assignment of the two peaks to the corresponding isomeric structures is not possible.

3.3. Validation

Total silibinin

Table 1 presents data of the inter-day accuracy and precision of each diastereomer determined from the daily calibration lines over a period of 23 days. The intra-day reproducibility of the assay based on repeated ($n = 7$) measurements at two concentrations (100 and 12.5 ng/ml) is shown in Table 2. An average correlation coefficient of 0.9977 ± 0.017 or better for both isomers confirmed the linearity of the calibration within the concentration range examined. The intra- and inter-day precision and accuracy, calculated as the coefficient of variation (C.V. %) and the percentage deviation from the true value (R.E. %) respectively, were generally below 10%, underlining the reliability of the assay. Only the 5 ng/ml calibration standard of diastereomer 2 revealed an inter-day C.V. of 15% and an R.E. of 10.8% which is, however, clearly below the cutoff criterion of ±20% precision and of ±15% accuracy.

Free silibinin

Table 3 presents data of the inter-day accuracy and precision of each diastereomer obtained from the daily calibration lines during 21 days.

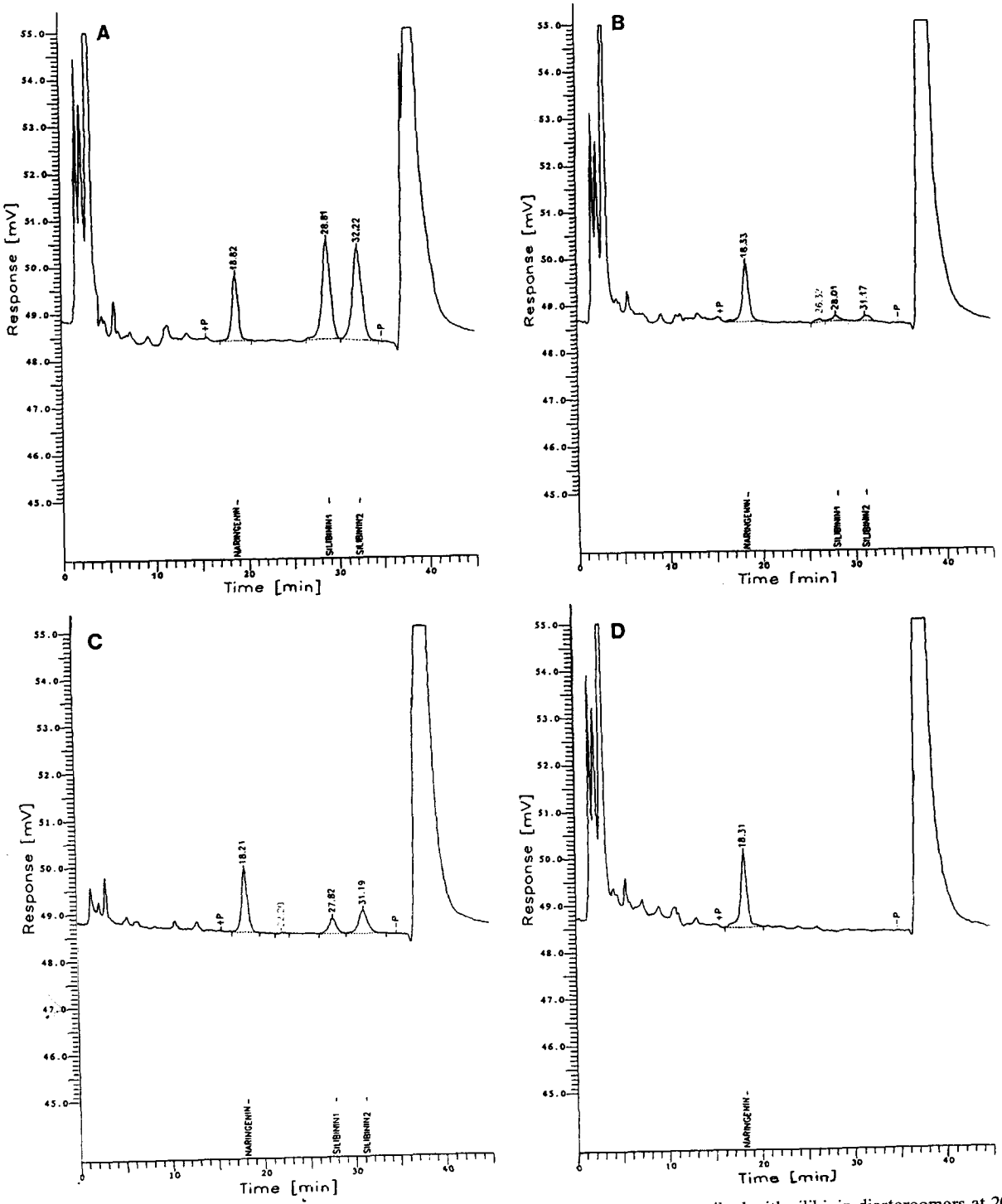

Fig. 3. Chromatograms of total (free + conjugated) silibinin diastereomers. (A) Plasma spiked with silibinin diastereomers at 200 ng/ml (=400 ng/ml silibinin), (B) plasma spiked with silibinin diastereomers at 12.5 ng/ml (=25 ng/ml silibinin), (C) plasma volunteer F.H. 1 h after a single oral dose of 140 mg of silymarin (=53.2 mg silibinin), plasma volume 20 μl: found 34.55 ng of diastereomer 1 and 55.13 ng of diastereomer 2, (D) blank plasma with internal standard naringenin. Chromatographic conditions as described in Section 2.4.

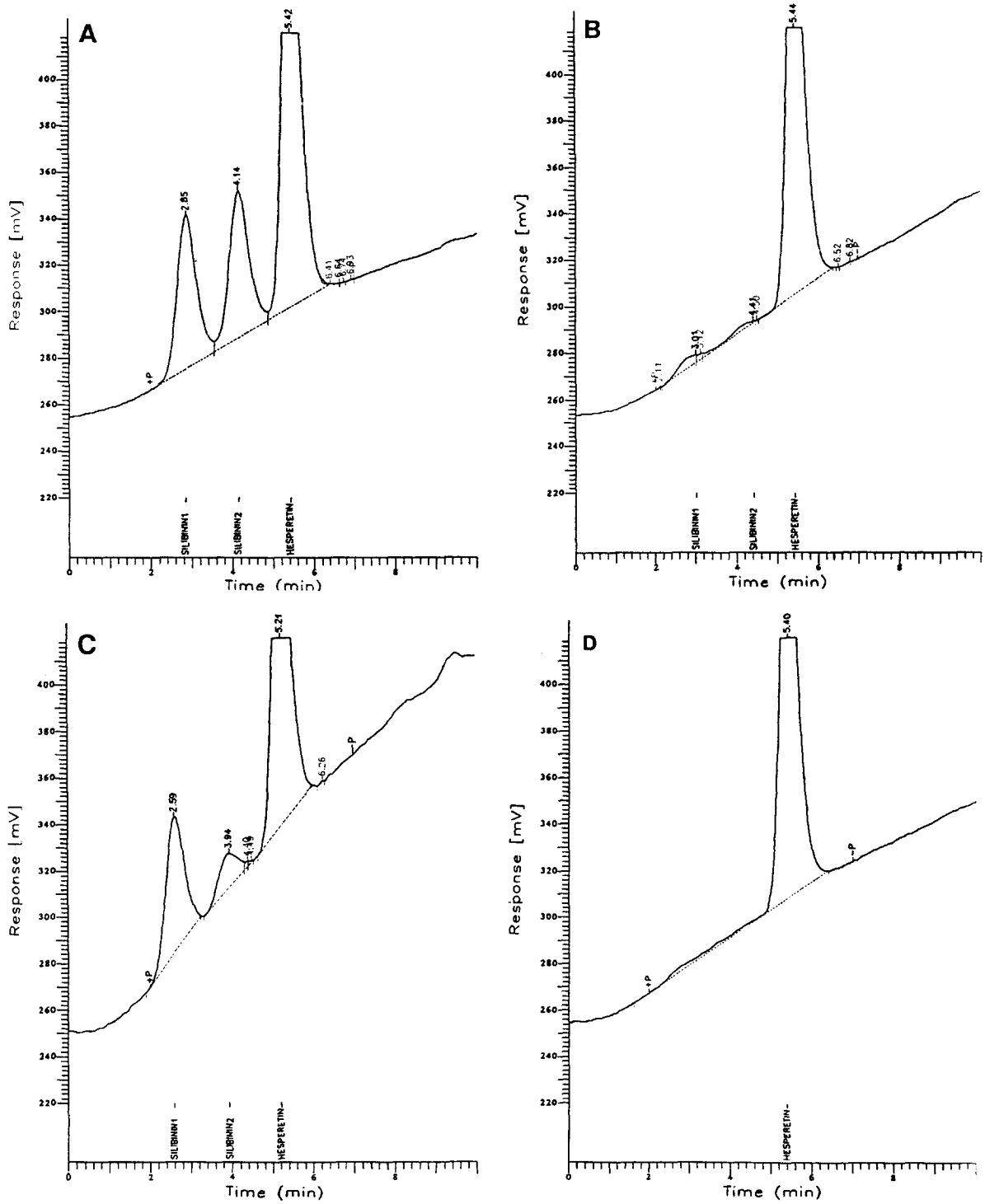

Fig. 4. Chromatograms of free silibinin diastereomers. (A) Plasma spiked with silibinin diastereomers at 5 ng/ml (=10 ng/ml silibinin), (B) plasma spiked with silibinin diastereomers at 0.25 ng/ml (=0.5 ng/ml silibinin), (C) plasma volunteer R.H. 4 h after a single oral dose of 140 mg of silymarin (=53.2 mg silibinin), found 6.49 ng/ml of diastereomer 1 and 1.77 ng/ml of diastereomer 2, (D) blank plasma with internal standard hesperetin. Chromatographic conditions as described in Section 2.3.

Table 1
Inter-day ($n = 23$) precision and accuracy of total (free and conjugated) silibinin diastereomers from spiked plasma samples after enzymatic hydrolysis

	Silibinin concentration added per diastereomer (ng/ml)					
	200	100	50	25	12.5	5
Diastereomer 1 (total)						
Mean silibinin found (ng/ml)	202.27	97.06	49.71	25.26	12.92	4.91
R.E. (%)	1.14	−2.94	−0.58	1.04	3.36	−1.80
S.D. (ng/ml)	3.34	3.13	2.76	2.03	0.99	0.34
C.V. (%)	1.65	3.22	5.55	8.02	7.69	6.92
Diastereomer 2 (total)						
Mean silibinin found (ng/ml)	201.40	96.71	50.20	25.81	12.97	5.54
R.E. (%)	0.70	−3.29	0.40	3.24	3.76	10.80
S.D. (ng/ml)	1.41	3.17	1.91	1.52	1.29	0.83
C.V. (%)	0.70	3.28	3.81	5.88	9.90	15.05

The intra-day reproducibility of the assay based on repeated ($n = 7$) measurements at three concentrations (25, 5 and 0.5 ng/ml) is shown in Table 2. The average correlation coefficient of 0.9988 ± 0009 or better for both isomers confirmed the linearity of the method within the concentration range examined. Except for the 0.5 ng/ml standard (intra-day R.E. 12%) the inter- and intra-assay precision (R.E.) and accuracy (C.V.) was generally below 10% outlining the reliability of the applied column-switching technique.

Table 2
Intra-day ($n = 7$) precision and accuracy of free (unconjugated) and total (free and conjugated) silibinin diastereomers from spiked plasma samples

	Silibinin diastereomer added (ng/ml)				
	Free silibinin			Total silibinin	
	25	5	0.5	100	12.5
Diastereomer 1					
Mean silibinin found (ng/ml)	24.93	4.95	0.53	102.79	11.95
R.E. (%)	−0.28	−1.00	6.00	2.79	−4.40
±S.D. (ng/ml)	0.82	0.26	0.07	3.16	0.88
C.V. (%)	3.28	5.18	12.74	3.08	7.39
Diastereomer 2					
Mean silibinin found (ng/ml)	25.51	4.92	0.56	100.07	11.33
R.E. (%)	2.04	−1.60	12.00	0.07	−9.36
±S.D. (ng/ml)	0.94	0.28	0.03	4.39	0.96
C.V. (%)	3.67	5.69	5.87	4.39	8.45

3.4. Application of the assay in a pharmacokinetic study

Mean plasma concentration–time curves of free and total silibinin diastereomers after a single oral dose of 140 mg silymarin (=53.2 mg silibinin) to 10 volunteers are depicted in Fig. 5. The occurrence of several absorption peaks is possibly due to the enterohepatic circulation of the compound. The mean AUC values for free and total silibinin diastereomers are given in Table 4. They were four times higher for diastereomer 1 than for diastereomer 2. The ratio is reversed considering total (free + conjugated) silibinin plasma levels with diastereomer 2 revealing twice the concentration of diastereomer 1. From these data it can also be concluded that 23% of diastereomer 1 and 3% of diastereomer 2 found in plasma are in unconjugated form. The difference between both isomers may be explained by stereoselective metabolism, absorption, or distribution. Unfortunately the individual diastereomers are not available as pure compounds. Therefore, no definite assignment of the

Table 3
Inter-day ($n = 21$) precision and accuracy of free (unconjugated) silibinin diastereomers from spiked plasma samples

	Silibinin concentration added per diastereomer (ng/ml)							
	50	25	12.5	5	2.5	1.25	0.5	0.25
Diastereomer 1 (unconjugated)								
Mean silibinin found (ng/ml)	50.14	24.90	12.60	4.88	2.49	1.24	0.52	0.25
R.E. (%)	0.28	−0.40	0.80	−2.40	−0.40	−0.80	4.00	0.00
±S.D. (ng/ml)	0.99	0.79	0.43	0.26	0.14	0.07	0.03	0.02
C.V. (%)	1.98	3.18	3.44	5.35	5.57	5.77	6.39	7.32
Diastereomer 2 (unconjugated)								
Mean silibinin found (ng/ml)	50.61	24.84	12.45	4.82	2.47	1.24	0.52	0.26
R.E. (%)	1.22	−0.64	−0.40	−3.60	−1.20	−0.80	4.00	4.00
±S.D. (ng/ml)	0.86	0.62	0.42	0.23	0.13	0.06	0.03	0.02
C.V. (%)	1.69	2.49	3.37	4.80	5.22	4.71	5.86	8.05

peaks "1" and "2" to the corresponding stereospecific structure is possible. For the same reason no data on pharmacological activity ratios are available, but it is expected that the antioxidative potencies [5] should not be much different.

4. Conclusion

Two stereoselective HPLC assays were developed for the determination of free and total silibinin diastereomers in human plasma: free

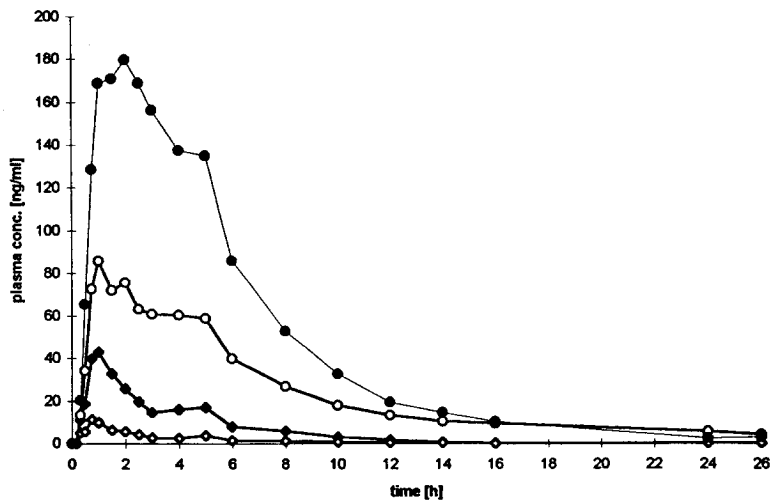

Fig. 5. Mean ($n = 10$) plasma concentration–time profiles of free and total silibinin diastereomers following administration of a single oral dose of 140 mg silymarin (=53.2 mg silibinin) to healthy volunteers. ◆ = Free diastereomer 1; ◇ = free diastereomer 2; ○ = total diastereomer 1; ● = total diastereomer 2.

Table 4
Mean AUC of free (unconjugated) and total (free and conjugated) silibinin diastereomers in 10 healthy volunteers after a single oral dose of 140 mg silymarin (=53.2 mg silibinin)

Compound	Diastereomer	AUC($0 - t_{last}$) (ng/ml · h)
Free silibinin	1	150.8 ± 59.3
	2	33.4 ± 14.8
Total silibinin	1	603.5 ± 257.5
	2	1193.0 ± 485.4

silibinin plasma concentrations were analyzed applying a column-switching HPLC method with electrochemical detection and total silibinin plasma levels were determined after enzymatic hydrolysis of the conjugates using an HPLC assay with UV detection.

Both methods are specific, robust and sensitive enough to quantify 0.25 ng/ml per diastereomer of free silibinin and 5 ng/ml of total silibinin respectively. They are more sensitive than the only stereospecific method [4] published previously. Additionally, the use of an internal standard provides more robustness. The described assays were successfully applied to the analysis of plasma samples from pharmacokinetic studies of silymarin in humans.

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