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Highly sensitive coupled-column high-performance liquid chromatographic method for the separation and quantitation of the diastereomers of leucovorin and 5-methyltetrahydrofolate in serum and urine

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Abstract

A column-switching chiral HPLC assay was developed that allows the separation and quantitation of the diastereomers of leucovorin (LV, 5-formyltetrahydrofolic acid) and its metabolite 5-methyltetrahydrofolate (METHF) in serum and urine by means of fluorescence detection. The analysis procedure consists of an on-line concentration of the folates in the HPLC system which is followed by the elution and separation of folates on an achiral 3-μm Microbore C_{18} column in (6*R*,*S*)-LV and (6*R*,*S*)-METHF. (6*R*,*S*)-LV and (6*R*,*S*)-METHF are subsequently transferred on-line onto a chiral 7-μm bovine serum albumin column through a Rheodyne valve system and are separated into their distereometers. Time of analysis is 70 min. Detection limit is 5 ng/ml for each diastereometer. The within-day variation ranges between 3.2 and 15.8% in relation to the measured concentration. Between-day variation is 4.4–12.1% for a concentration of 100 ng/ml for each diastereometer. (6*R*,*S*)-LV and (6*S*)-LV pharmacokinetics were assessed by analyzing serum and urine samples of four-healthy volunteers.

1. Introduction

Leucovorin (LV, 5-formyltetrahydrofolic acid) has become established as part of antineoplastic combination regimens for several malignant disorders. Hence, it is used for recue after high- and intermediate-dose methotrexate therapy and also as a modulator of the pyrimidine antagonist 5-fluoruracil (5-FU) [1]. The modulating effect of LV is thought to be primarily mediated through the stabilization of the ternary complex of the thymidilate-synthase, fluorodeoxyuridylate and folate cofactor. By this mechanism the antineoplastic activity of 5-FU is substantially enhanced as demonstrated by a variety of controlled clinical investigations [2–7]. LV originated from a chemical reduction, which is not stereospecific and therefore results in equal amounts of the diastereometers (6*R*)-LV and (6*S*)-LV [8].

The biological activity of LV is apparently restricted to the (6*S*)-LV diastereomer which is rapidly converted to its active metabolite (6*S*)-5-methyletrahydrofolate(METHF) while the (6*R*)-LV diastereomer is not metabolized and is excreted unchanged through the kidneys with a plasma half-live of about 7 h [9,10]. However,

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information about a potential interaction between both isomer forms and a modulation of (6*S*)-LV metabolism and efficacy by the (6*R*) diastereomer is sparse and suggests that (6*R*)-LV could inhibit the cellular uptake of (6*S*)-LV and (6*S*)-METHF. This issue has regained substantial importance through the availability of purified (6*S*)-LV and its evaluation in clinical phase I and II studies [11–13]. In order to elucidate the interaction between both diastereomers and to analyse their pharmacokinetics, a reliable and suitable HPLC method is needed.

As yet, the diastereometers of LV and METHF are quantitated mainly by microbiological techniques, which depend on the different metabolism of various folates by various bacterial species [14]. These assays are hampered by the lack of specificity due to a cross reactivity of bacterial strains with other folates and the interference of microorganisms with antifolates and antibiotics [15–18]. Furthermore, they are time-consuming and expensive and hence not suited for routine clinical measurements. In the last few years HPLC methods were developed that allow a separate analysis of the diastereomers of LV and METHF. These methods use a chiral BSA (bovine serum albumin) column for separation and require a complicated evaporation step [15,16,19,20]. Three of four published assays have a very low detection limit of 0.5–1 μg/ml plasma and are no real alternatives to microbiological assays which show a sensitivity of 0.2–1 ng/ml. For two of these methods no validation of key parameters like recovery, standard deviation and coefficient of variation has been reported [15,20]. The fourth available assay has a sufficient detection limit of 5 ng/ml for (6*R*)- and (6*S*)-LV but is limited by a time-consuming sample clean-up and an additional evaporation step [19]. In this assay the quantitation of all four diastereomers is not possible during one run. Furthermore, samples have to be switched from one chiral column to two achiral columns without visual control.

We here describe a sensitive and reproducible HPLC method for the quantitation of the diastereomers of LV and METHF in serum and urine which is based on an on-line concentration of the folates in the HPLC system, the separation of the folates on an achiral 3-μm Microbore C_{18} column in (6*R*,*S*)-LV and (6*R*,*S*)-METHF and the consecutive separation into the diastereometers (6*S*)-LV, (6*R*)-LV, (6*R*)-METHF and (6*S*)-METHF by a chiral 7-μm BSA column. All folate transfers in the HPLC system were performed on-line using three Rheodyne valves. The technique was used to determine pharmacokinetic parameters in four healthy volunteers after a short-time infusion of 200 mg/m^2 (6*R*,*S*)-LV and 100 mg.m^2 (6*S*)-LV after a wash-out phase of 4 days.

2. Experimental

2.1. Materials

5-METHF and LV were purchased from Sigma. All salts and ascorbic acid were obtained from E. Merck and were of analytical-reagent quality. (6*R*,*S*)-LV and (6*S*)-LV were kindly supplied by Ribosepharm (Haan, Germany). Acetonitrile, 2-propanol, phosphoric acid and water were of HPLC quality and were purchased from J.T. Baker, tetrabutyl ammonium phosphate (PIC A, low-UV reagent, TBAP) and ultra-filtration cartridges were obtained from Millipore/Waters, 1-ml C_{18} solid-phase extraction cartridges and 40-μm C_{18} silica material were purchased from Supelco.

2.2. HPLC apparatus

The chromatographic system consisted of 3 Jasco Model PU 880 analysis pumps (Jasco, Gross-Zimmern, Germany) and a low-pressure S 1311 reagent-dosing pump from Sykam (Gilching, Germany), a Jasco 820 FP fluorimetric detector with an optimum signal for emission at 365 nm and for excitation of 308 nm, a Rheodyne injection valve 7125 with a 5-ml sample loop and three Rheodyne valves 700 for column switching. Valve C (transfer valve, Fig. 1) was equipped with a 1-ml sample loop. The chiral BSA column and the achiral Microbore C_{18} column were heated to 40°C with an incubator.

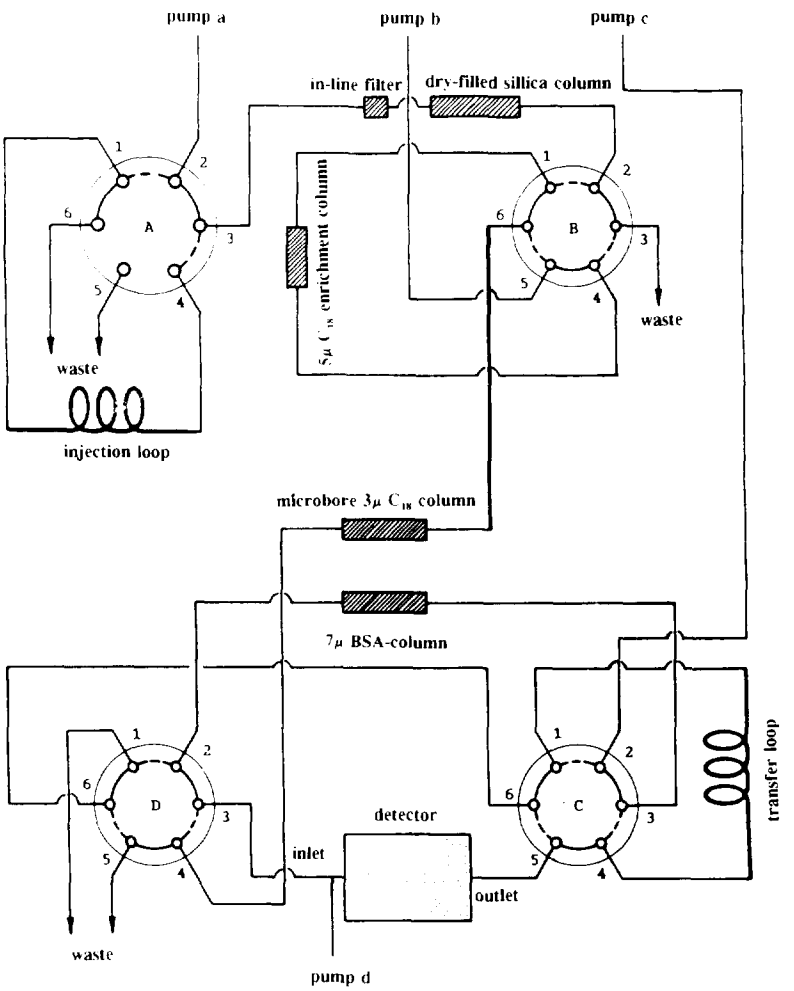

Fig. 1. Valve system diagram for HPLC separation of (6*R*)-LV, (6*S*)-LV, (6*R*)-METHF and (6*S*)-METHF. A = injection valve; B = enrichment valve; C = transfer valve; D = detector switching valve.

Evaluation and quantitation of chromatograms was done by a PC supported NINA program of Nucleare Interface (Münster, Germany).

2.3. HPLC conditions

For the enrichment of the folates a Macherey & Nagel 30 × 4 mm I.D. cartridge with 5-μm octadecyl silica material was used. A 5-μm in-line filter and a 125 × 4 mm I.D. cartridge, dry-filled with 40-μm silica material, were applied to increase the life of the enrichment column, to retain hydrophilic substances and to obtain a better mixture of the samples with the eluent. The eluent for enrichment (eluent A, pump a, Fig. 1) contained 0.005 *M* TBAP, adjusted to a pH of 6.5 with H_3PO_4, the flow was 2 ml/min and the enrichment column temperature was ambient. The chromatographic separation on the achiral part of the system was performed on a Macherey & Nagel Microbore 250 × 2 mm I.D. 3-μm C_{18} column. The mobile phase (eluent B, pump b, Fig. 1) was composed of sodium phosphate (0.0015 *M*), TBAP (0.00075 *M*), and 2-propanol (7.5%, v/v), pH 5 adjusted with H_3PO_4. The flow-rate was 0.15 ml/min and the

temperature 40°C. A Resolvosil 7-μm 150 × 4.6 mm I.D. BSA-7 column was used for the chiral part of the system. It was flushed with 0.028 *M* phosphate buffer containing 0.0006 *M* sodium azide for protection of the BSA against growth of microorganisms (eluent C, pump c, Fig. 1). The flow was 0.4 ml/min and the column temperature was maintained at 40°C. Since (6*R*,*S*)-METHF showed a pH-dependent reciprocal fluorescence emission, water adjusted to pH 1.5 with phosphoric acid (eluent D) was mixed to the flow of eluent B by a reagent dosing pump which was connected between the detector switching valve and the fluorescence detector. Flow of this pump (pump d, Fig. 1.) was adjusted to 0.12 ml/min. This on-line acidification to a pH of 2 led to an approximately 16-fold increase in the signal of (6*R*,*S*)-METHF compared to the signal at pH 5. Since (6*R*,*S*)-LV was degraded under these conditions, the eluent acidification by pump d was only used for the C_{18} retention time of (6*R*,*S*)-METHF.

2.4. Valve-switching system

Fig. 1 shows the switching system realized by the use of four Rheodyne valves. Table 1 shows the sequence of valve positions and the resulting flow direction. Folates of injected samples were enriched on a 5-μm C_{18} column while serum constituents such as proteins and salts were eluted. Then valve B is switched and the folates are eluted retrogradely from the enrichment column by the flow of eluent B produced by pump b. On the analytical 250 × 2 mm I.D., 3-μm C_{18} colum the separation of the folates in (6*R*,*S*)-LV and (6*R*,*S*)-METHF is registered by fluorescence detection. When the (6*R*,*S*)-LV peak is in the 1-ml transfer loop of valve C, it is switched onto the chiral column by flow of pump c. Time for switching value C can be calculated by $T = (V_1 + V_2)/F - B$, where T is the retardation time in min, V_1 the detector cell volume in μl, V_2 the outlet capillary volume, F the flow of eluent C in μl/min and B the half peak width in min. For our system a retardation time of 55 s after peak maximum of (6*R*,*S*)-LV was calculated. On the BSA column (6*R*,*S*)-LV is separated into its diastereomers which are again registered by fluorescence detection. Valve D spares the use of a second detector because it allows to switch the detector either in series with the analytical C_{18} column or in series with the BSA column. Because of an t_R difference of 4 min between (6*R*,*S*)-LV and (6*R*,*S*)-METHF and a flow of 0.4 ml/min on the BSA column, valve C can be switched again to the origin position after 3.5 min. At this point pump d is started for acidification of eluent B to improve the detector signal of (6*R*,*S*)-METHF. When (6*R*,*S*)-METHF is in the transfer loop, valve C is switched again 55 s after its peak maximum. Finally, the detector is connected by valve D in series to the BSA column, so that all four diastereometers can be determined when they are eluted from the 7-μm BSA column.

2.5. Sample preparation

All samples are stabilized with ascorbic acid (0.5 mg/ml). After addition of acetonitrile (plasma–acetonitrile 1:1.5, v/v), the samples are

Table 1
Sequence of valve positions and description of direction of flow

Step during analysis	Flow of pump A	Flow of pump B	Flow of pump C
Enrichement of folates	A2-1-4-3,B2-1-4-3	B5-6,D4-3,C5-4-1-6,D6-5	C2-3,D2-1
Elution of folates from enrichment column and separation on the achiral C_{18} column	A2-1-4-3,B2-3	B5-4-1-6,D4-3,C5-4-1-6,D6-5	C2-3,D2-1
Transfer of (6*R*,*S*)-LV to BSA column	A2-1-4-3,B2-3	B5-4-1-6,D4-3,C5-6, D6-5	C2-1-4-3,D2-1
Valve C is switched again	A2-1-4-3,B2-3	B5-4-1-6,D4-3,C5-4-1-6,D6-5	C2-3,D2-1
Transfer of (6*R*,*S*)-METHF to BSA column	A2-1-4-3,B2-3	B5-4-1-6,D4-3,C5-6, D6-5	C2-1-4-3,D2-1
Detector is switched in line to BSA column	A2-1-4-3,B2-3	B5-4-1-6,D4-5	C2-1-4-3,D2-3C5-6D6-1

vortex-mixed and centrifuged (400 *g*) for 1 min. For extraction of acetonitrile 7 ml chloroform are added to the supernatant. After mixing, this solution is again centrifuged (400 *g*) for 1 min. A maximum of 400 μl of the aqueous phase is diluted with eluent A (1:5, v/v) and injected. At the end of the sample clean-up 400 μl of the aqueous phase corresponds to 400 μl serum. Urine samples are diluted with eleuent A (1:4) and are enriched on 1-ml Supelco C_{18} solid-phase extraction cartridges, which are activated by 2 ml water, 2 ml methanol and 2 ml of eluent A. Matrix substances are rinsed off by washing the cartridges with 2 ml of eleuent A. Elution of folates was carried out with 2 ml of 50% acetonitrile. After extraction of acetonitrile with chloroform, the aqueous phase is centrifuged through ultra-filtration cartridges. An aliquot of 400 μl is diluted with eluent A (ratio 1:5) and injected for quantitation. This preparation procedure for urine is only necessary for folate concentrations <500 ng/ml. For higher concentrations 50 μl urine is mixed with 500 μl eluent A and injected without further preparation into the enrichment system of the HPLC.

2.6. Quantitation of (6S)-LV, (6R)-LV, (6S)-METHF and (6R)-METHF

The diastereomers of (6*R*,*S*)-LV and (6*R*,*S*)-METHF are quantified using the external standard method by plotting the peak areas from aqueous standards with 5, 50, 100, 250, 500, 750, 1000 and 2000 ng diastereomer/ml against the concentrations. The calibration data are fitted to a linear, unweighted, forced-through-zero model computed by the calibration function of the PC chromatography programme (NINA). The resultant linear regression curves is used to calculate the concentration of each diastereomer in the samples.

2.7. Method application

The application of the described analytical procedures was proven on plasma and urine samples from four healthy volunteers after application of 200 mg/m^2 (6*R*,*S*)-LV and 100 mg/m^2 (6*S*)-LV by a 10-min infusion. A wash-out phase of 4 days separated the infusions of (6*R*,*S*)-LV and (6*S*)-LV. Plasma samples were collected before and 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h after LV infusion. Urine was analysed before and 4, 8, 12 and 24 h after LV infusion. Ascorbic acid (1 mg/ml) was added to serum and urine samples as antioxidant prior to storage at −20°C. Pharmacokinetic parameters were determined by the "Topfit" pharmacokinetic computer program using a linear two-compartment model for fitting the measured data [21,22].

3. Results

3.1. Separation parameters

Chromatographic parameters for the separation of the four diastereomers on the BSA column gained from spiked plasma (Fig. 3b) are depicted in Table 2. They demonstrate the well

Table 2
Results of chromatographic parameters

Compound	k'	R	T	N	H (μm)
(6*S*)-LV	1.85		2.28	284	528
		1.56			
(6*R*)-LV	2.98		1.55	249	602
(6*R*)-METHF	4.63		2.73	217	691
		3.17			
(6*S*)-METHF	8.02		2.76	530	283

k' = Capacity factor; R = resolution factor; T = tailing factor; N = efficiency; H = HETP, height equivalent to a theoretical plate.

known low efficiency but good resolution of the chiral BSA column.

3.2. Specificity and identification of the diastereomers of LV and METHF

In 10 plasma and urine samples of 10 healthy volunteers no coeluting peaks for the diastereomers were detected (Fig. 2c). After spiking the samples prior to preparation with 50 ng/ml for each diastereomer, 6 new peaks with retention times of (6*R*,*S*)-LV, (6*R*,*S*)-METHF, (6*R*)-LV, (6*S*)-LV, (6*R*)-METHF and (6*S*)-METHF in aqueous standards could be identified (Fig. 3a,b). Addition of only one isomer to a blank serum sample showed a new peak, whose retention time corresponded with the retention time of the appropriate peak of an aqueous standard. Furthermore, retention times of analyzed serum samples of healthy volunteers who had received a LV infusion corresponded to the retention times of (6*R*)-LV, (6*S*)-LV and (6*S*)-METHF (Fig. 2a). The elution order that was found was (6*S*)-LV, (6*R*)-LV, (6*R*)-METHF, (6*S*)-METHF and corresponded with the elution order described in another publication [15].

3.3. Linearity and recovery

The linearity of the method was proven from 5 ng/ml to 2000 ng/ml in spiked plasma samples and spiked urine at 8 known concentrations (5 ng/ml, 50 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml, 750 ng/ml, 1000 ng/ml and 2000 ng diastereomer/ml) and was evaluated by the linear regression analysis method (Fig. 4). The calculated correlation coefficients were 0.999 for (6*R*)-LV, (6*S*)-LV and (6*R*)-METHF and 0.998 for (6*S*)-METHF in plasma and 0.988 for (6*R*)-LV, 0.995 for (6*S*)-LV, 0.999 for (6*R*)-METHF and 0.978 for (6*S*)-METHF in urine.

3.4. Within-day variation and recovery

To evaluate the within-day variation of folate measurements, ten analyses at four different concentrations (5, 50, 200 and 500 ng diastereomer/ml) were performed for plasma and urine.

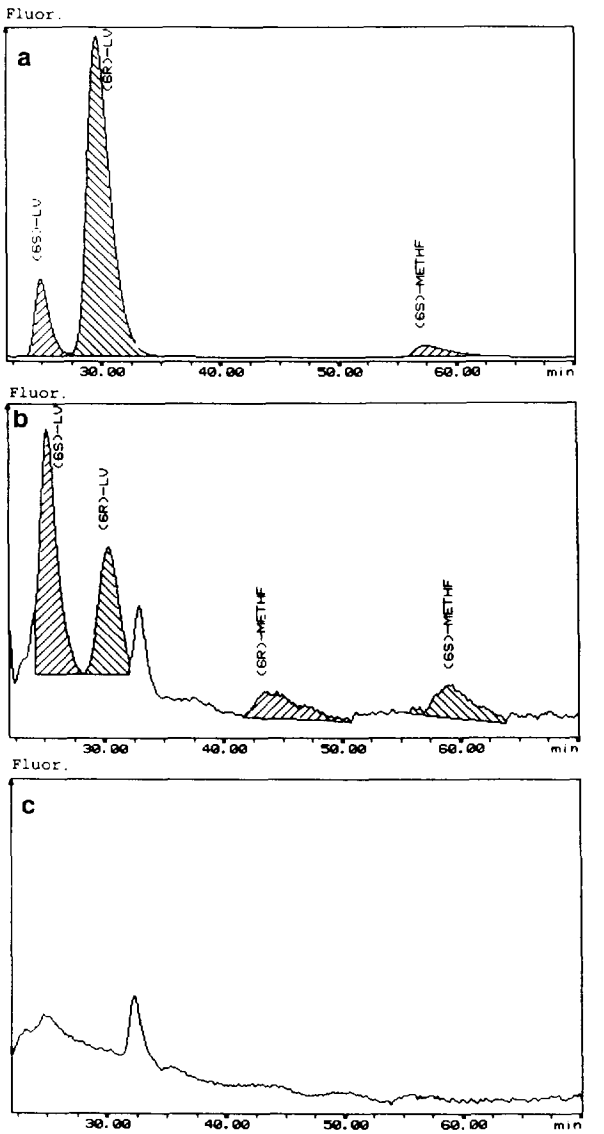

Fig. 2. BSA column separation of (a) 60 μl plasma from a healthy volunteer taken 2 h after application of 200 mg/m^2 (6*R*,*S*)-LV, (b) 400 μl spiked plasma with 5 ng diastereomer/ml, (c) 400 μl blank plasma (microbore C_{18} separation now shown).

For each sample 400 μl plasma and 400 μl urine were prepared. Recovery, calculated average, standard deviation (S.D.) and the coefficient of variation (C.V.) of these measurements are presented in Tables 3 and 4 and show a concentration-dependent standard devaition of 3.2–

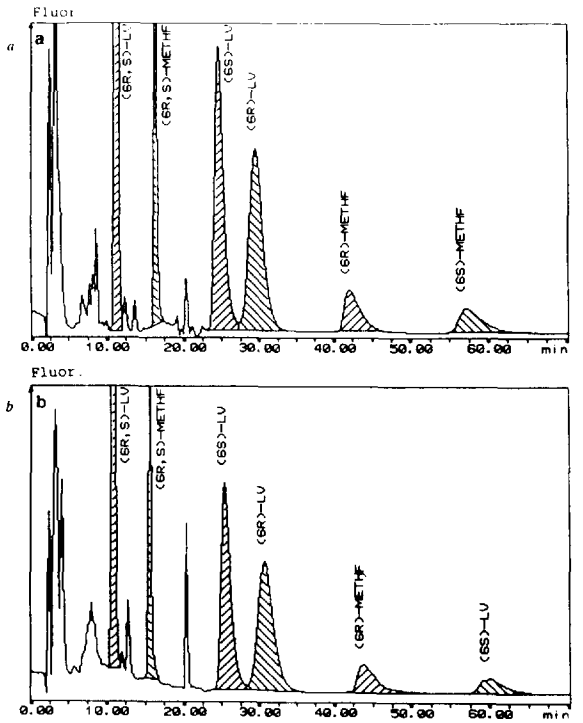

Fig. 3. HPLC separation on microbore C_{18} and BSA column of (a) 400 μl aqueous standard with 50 ng diastereomer/ml, (b) 400 μl spiked plasma, 50 ng diastereomer/ml.

15.8%. Recovery was concentration independent and ranged from 72–89% for (6*S*)-LV, 70–84% for (6*R*)-LV, 78–94% for (6*R*)-METHF and 76–90% for (6*S*)-METHF. There was no significant difference for these parameters in plasma or urine samples.

3.5. Between-day variation and stability

Between-day variation studies were performed by analyzing 400 μl plasma and urine samples at ten consecutive days. These samples contained 100 ng (6*R*)-LV, 100 ng (6*S*)-LV, 100 ng (6*R*)-METHF and 100 ng (6*S*)-METHF. Oxidation of folates which would be caused by repeated thawing and freezing of samples was avoided by the following procedure. In the beginning 10 ml serum and urine were adjusted to a concentration of 1 mg/ml ascorbic acid. These samples were spiked with 100 ng/ml for each diastereomer and were then distributed to 25 Eppendorf vials which were immediately frozen. By this approach samples were not thawed before the day of analysis. These samples were used furthermore for quality control over a period of 3 months and no significant oxidative degradation of folates during storage at −20°C was found. Results of between-day variation for plasma and urine are presented in Tables 5 and 6 and show in general the same results as for the within-day variation.

3.6. Detection limit

A peak with three- to sixfold the height of the average noise on the BSA column was taken as the detection limit, because peak widths of 7 min [for (6*R*)-METHF and (6*S*)-METHF] did not allow an exact integration of peaks with twice the height of the average noise. A representative chromatogram from serum spiked with 5 ng/ml for each diastereomer is presented in fig. 2b. After separation on the microbore C_{18} column, an approximately 4-fold lower detection limit could be reached for the unseparated diastereomers as depicted in Fig. 5a and b, especially for (6*R*,*S*)-METHF using acidification of the eluent by the reagent dosing pump d. This procedure may also improve the detection limit for (6*R*)-METHF and (6*S*)-METHF after BSA column separation, but it will also enlarge the peak volume, however, and requires a low pH, respectively, a higher flow than used in eluent D due to the higher flow-rate on the BSA column. Therefore, and because of the good detection limit of 5 ng/ml for (6*R*)-METHF and (6*S*)-METHF, acidification after BSA separation was not proven.

3.7. Pharmakokinetics of (6R,S)-LV and (6S)-LV

Fig. 6 shows a representative plasma concentration–time profile and the cumulative urine excretion of the diastereomers (6*R*)-LV, (6*S*)-LV and (6*S*)-METHF in a healthy volunteer after a 10-min infusion of 200 mg/m^2 (6*R*,*S*)-LV fitted to a two-compartment model by the "topfit"

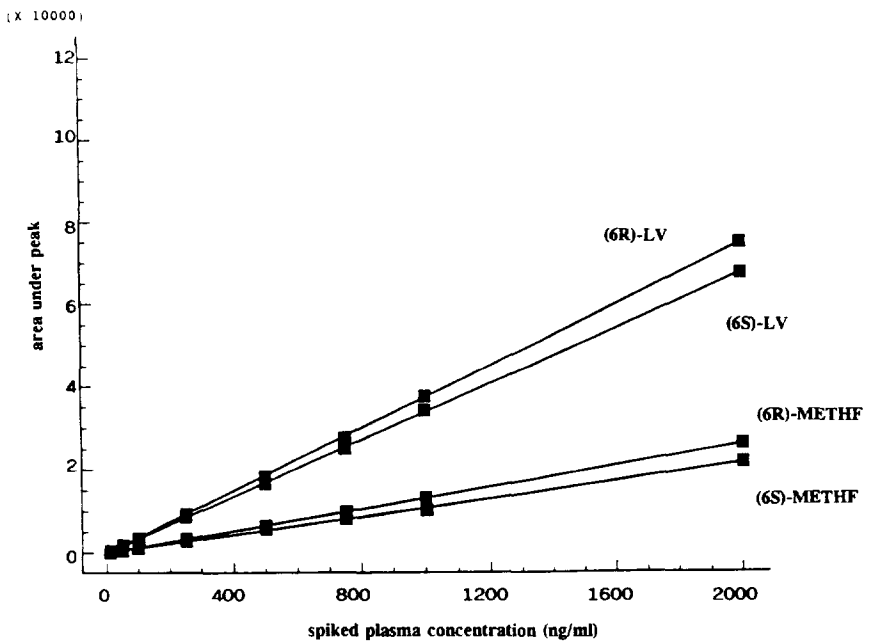

Fig. 4. Area-under-peak to concentration regression curve analysed from spiked plasma for (6*R*)-LV, (6*S*)-LV, (6*R*)-METHF and (6*S*)-METHF.

Table 3
Within-day validation in plasma

Compound	*n*	Analysed concentration (mean ± S.D.) (ng/ml)	Recovery (%)	C.V. (%)
500 ng/ml plasma				
(6*S*)-LV	10	407 ± 14.0	81	3.4
(6*R*)-LV	10	388 ± 38.2	78	9.8
(6*R*)-METHF	10	438 ± 19.3	88	4.4
(6*S*)-METHF	10	410 ± 27.2	82	6.7
200 ng/ml plasma				
(6*S*)-LV	10	179 ± 7.22	89	3.9
(6*R*)-LV	10	160 ± 7.83	80	4.8
(6*R*)-METHF	10	189 ± 8.46	94	4.5
(6*S*)-METHF	10	174 ± 8.21	87	4.7
50 ng/ml plasma				
(6*S*)-LV	10	36 ± 2.4	72	6.7
(6*R*)-LV	10	39 ± 5.8	78	14.8
(6*R*)-METHF	10	42 ± 2.1	84	5.0
(6*S*)-METHF	10	41 ± 2.8	82	6.9
5 ng/ml plasma				
(6*S*)-LV	10	4.3 ± 0.4	86	9.3
(6*R*)-LV	10	3.7 ± 0.3	74	8.1
(6*R*)-METHF	10	3.9 ± 0.4	78	10.3
(6*S*)-METHF	10	4.5 ± 0.6	90	13.3

Table 4
Within-day validation in urine

Compound	*n*	Analysed concentration (mean ± S.D.) (ng/ml)	Recovery (%)	C.V. (%)
500 ng/ml urine				
(6*S*)-LV	10	378 ± 12.2	76	3.2
(6*R*)-LV	10	401 ± 28.0	80	7.0
(6*R*)-METHF	10	390 ± 23.5	78	4.3
(6*S*)-METHF	10	417 ± 24.8	83	6.0
200 ng/ml urine				
(6*S*)-LV	10	176 ± 8.0	88	4.5
(6*R*)-LV	10	167 ± 6.5	84	3.9
(6*R*)-METHF	10	175 ± 5.7	88	3.3
(6*S*)-METHF	10	180 ± 7.1	90	4.0
50 ng/ml urine				
(6*S*)-LV	10	39 ± 3.7	78	9.5
(6*R*)-LV	10	35 ± 4.1	70	11.7
(6*R*)-METHF	10	43 ± 3.4	86	7.9
(6*S*)-METHF	10	38 ± 3.9	76	10.2
5 ng/ml urine				
(6*S*)-LV	10	3.8 ± 0.6	76	15.8
(6*R*)-LV	10	4.1 ± 0.4	82	9.8
(6*R*)-METHF	10	4.0 ± 0.5	80	12.5
(6*S*)-METHF	10	3.9 ± 0.5	78	12.8

pharmakokinetic computer programme. Average pharmacokinetic parameters from four applications of 200 mg/m^2 (6*R*,*S*)-LV and 100 mg/m^2 (6*S*)-LV to four healthy volunteers are depicted in Table 6. No significant differences between (6*R*,*S*)-LV and (6*S*)-LV were found in dose-dependent or in dose-independent kinetic parameters.

Table 5
Between-day validation in plasma and urine

Compound	*n*	Analysed concentration (mean ± S.D.) (ng/ml)	Recovery (%)	C.V. (%)
100 ng/ml plasma				
(6*S*)-LV	10	74 ± 4.4	74	6.0
(6*R*)-LV	10	66 ± 5.5	66	8.4
(6*R*)-METHF	10	73 ± 8.8	73	12.1
(6*S*)-METHF	10	74 ± 3.3	74	4.4
100 ng/ml urine				
(6*S*)-LV	10	77 ± 5.6	77	7.3
(6*R*)-LV	10	72 ± 8.1	72	11.3
(6*R*)-METHF	10	80 ± 6.3	80	7.9
(6*S*)-METHF	10	76 ± 5.9	76	7.8

Table 6
Pharmakokinetic parameters from four healthy volunteers after application of 200 mg/m^2 (6*R*,*S*)-LV and 100 mg/m^2 (6*S*)-LV

$n = 4$ 200 mg/m^2 (6*R*,*S*)-LV and 100 mg/m^2 (6*S*)-LV	$t_{1/2\beta}$ (h)	AUC (μg h/ml)	C_{max} (μg/ml)	t_{max} (h)	Cl_{tot} (ml/min)	Cl_{ren} (ml/min)	$Dosis_{ren}$ (%)	V_{ss} (l)
(6*S*)-LV	1.2	10	11.8	–	306	108	33	19
(after (6*R*,*S*)-LV application)	(30%)	(25%)	(34%)		(31%)	(52%)	(21%)	(21%)
(6*S*)-LV	1.2	11	11.5	–	285	97	34	19%
(after (6*S*)-LV application)	(17%)	(25%)	(19%)		(33%)	(37%)	(8%)	(14%)
(6*S*)-METHF	4.1	19	2.5	2.2	110	96	57	32
(after (6*R*,*S*)-LV application)	(42%)	(33%)	(28%)	(19%)	(30%)	(25%)	(18%)	(40%)
(6*S*)-METHF	3.9	20	2.5	2.5	100	89	57	29
(after (6*S*)-LV application)	(31%)	(23%)	(17%)	(19%)	(25%)	(25%)	(12%)	(28%)
(6*R*)-LV	6.7	134	24		23	16	69	12
(after (6*R*,*S*)-LV application)	(19%)	(17%)	(39%)		(23%)	(52%)	(34%)	(34%)

Values in parentheses are coefficients of variation.

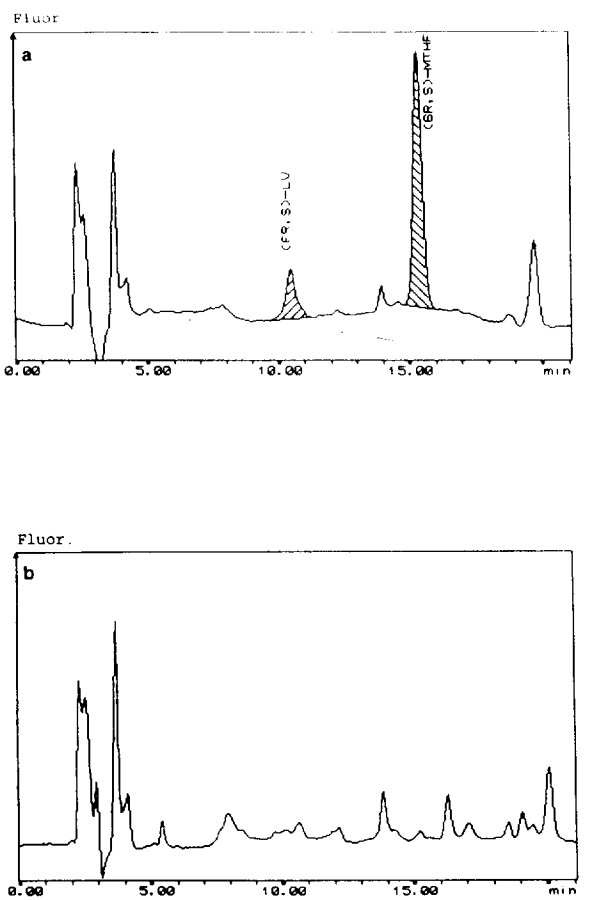

Fig. 5. Microbore C_{18} separation of (a) 400 μl spiked plasma with 10 ng/ml (6*R*,*S*)-LV and 10 ng/ml (6*R*,*S*)-METHF, (b) 400 μl blank plasma (BSA column separation not shown).

4. Discussion

As the BSA column with its low efficiency is the only known column for the separation of the diastereomers of LV and METHF at present, a sensitive separation of the stereoisomers in the low range can only be acehived at a certain mechanical and technical expense. Thus, the method presented here comprises three isocratic separate systems —an enrichment system, an achiral and a chiral system— which are set in motion by three separate pumps and are connected by three six-port valves. As to the detection limit and chromatographic parameters only the method by Silan et al. [19] is an alternative to this assay. The technique applied by Silan et al. is based on a chiral and achiral component, the latter consisting of two columns. Two pumps provide for the flow of the eluents on the achiral columns, a gradient elution is set in motion whose components are changed six times during the 70 min of analysis. The transfer of the substances and eluents is made by a ten-port and six-port valve. Our method, however, has important advantages at approximately the same mechanical and technical expense. In contrast to Silan et al.'s method there is a visual possibility of control for the peaks, both on the achiral as on the chiral column. This allows an immediate reaction at every possible t_R-timeshift by adapting the valve operations to the new conditions.

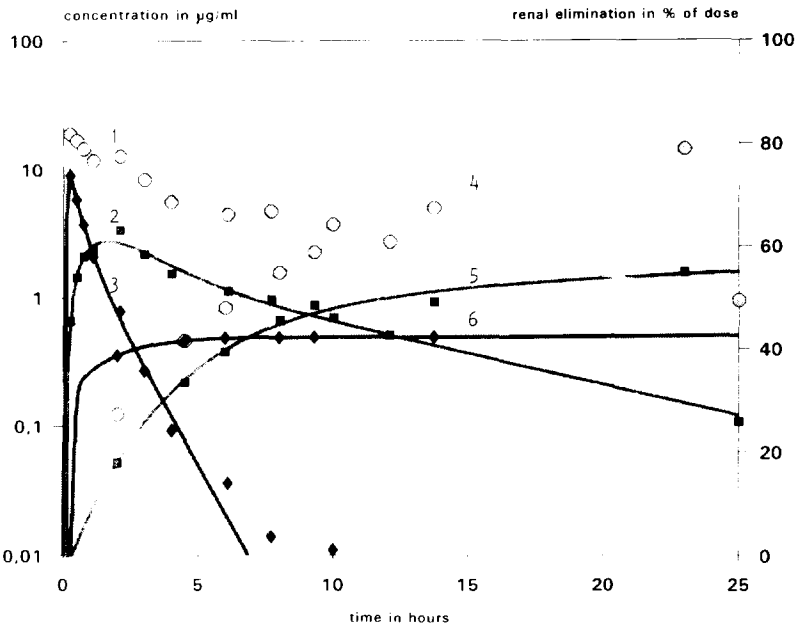

Fig. 6. Plasma concentration–time profiles and cumulative renal elimination of folates from a healthy volunteer after 10 min i.v. infusion of 200 mg/m² (6*R*,*S*)-LV. Plasma: 1 = (6*R*)-LV; 2 = (6*S*)-METHF; 3 = (6*S*)-LV; urine: 4 = (6*R*)-LV; 5 = (6*S*)-METHF; 6 = (6*S*)-LV.

As in Silan et al.'s method the substance peaks are transferred to the achiral C_{18} columns without visual control making a frequent control of the RT-times of the diastereometers on the BSA column mandatory. Therefore there is a constant uncertainty as to the fact what is really measured. Silan et al. describe a recovery of 100% in their assay. In view of the fact that a complex preparation procedure is applied comprising the coagulation of plasma proteins with acetonitrile, a 15-min centrifugation of plasma proteins, an evaporation of the sample volume, followed by a reconstruction of the residue in ascrobic acid and a final centrifugation by a micro-partition system, a total recovery of the labile folates must be questioned and is not confirmed by the current data. The assay presented here allows the separation and quantitation of all four diastereomers —(6*R*)-LV, (6*S*)-LV, (6*R*)-METHF and (6*S*)-METHF— within a 70-min analysis. None of the known methods offers that possibility. This method is hence an alternative to the variety of existing achiral assays for (6*R*,*S*)-LV and (6*R*,*S*)-METHF, and can be carried out without changing the system in a short time of analysis (20 min) with a much lower detection limit especially for (6*R*,*S*)-METHF [11,23,24].

The comparison of (6*R*,*S*)-LV and (6*S*)-LV pharmakokinetics in four healthy volunteers analysed by this HPLC method is in good correspondence with the few published results [13,25]. The new HPLC assay may thus be used in the context of clinical studies with (6*R*,*S*)-LV and (6*S*)-LV and could be the basis for analysing the intracellular metabolism of folates which will improve our knowledge of 5-FU modulation by leucovorin.

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