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Determination of low levels of the stereoisomers of leucovorin and 5-methyltetrahydrofolate in plasma using a coupled chiral–achiral high-performance liquid chromatographic system with post-chiral column peak compression

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ABSTRACT

A method for the determination of low levels of the stereoisomers of leucovorin and 5-methyltetra-hydrofolate has been developed and validated. The assay involved initial chromatography on a bovine serum albumin (BSA)-based high-performance liquid chromatography chiral stationary phase (CSP) followed by post-column peak compression and elution on two C_{18} columns In this manner, the poor efficiency of the BSA-CSP was overcome and sub-microgram quantities of the target solutes could be detected The BSA-CSP separated the leucovorin and 5-methyltetrahydrofolate from interfering plasma components and from each other and achieved the stereochemical resolution of the diastereomeric (6S)- and (6R)-leucovorin. The eluent containing (6S)-leucovorin was directed onto one C_{18} column and the eluent containing (6R)-leucovorin and 5-methyltetrahydrofolate was directed onto the other This was followed by sequential rapid gradient elution of the target compounds from the respective C_{18} columns The method was validated for plasma levels ranging from 15 to 500 ng/ml and was able to detect leucovorin concentrations of as low as 5 ng/ml.

INTRODUCTION

We have previously reported a coupled achiral–chiral high-performance liquid chromatographic (HPLC) method for the determination in plasma of the stereoisomers of leucovorin (LV), (6S)-LV and (6R)-LV, and the major metabolite

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(6*S*)-5-methyltetrahydrofolate (5-METHF) [1]. In this approach, (6*R*,*S*)-LV and 5-METHF were separated from interfering plasma peaks and quantified on a phenyl columnn and then selectively switched to a column containing an immobilized bovine serum albumin (BSA)-based chiral stationary phase (CSP) for the determination of the stereoisomeric composition. This method was able to detect (6*S*)- and (6*R*)-LV plasma concentrations of as low as 50 ng/ml, but could only be validated for plasma concentrations of 1 μg/ml and higher [2].

The major difficulty in the validation of sub-microgram quantities of LV and 5-METHF was the low efficiency of the BSA-CSP. At the low end of the concentration curves, the LV and 5-METHF peaks tended to merge with the background noise in the chromatogram. Since (6*S*)- and (6*R*)-LV are diastereomers, it is theoretically possible to separate them using an achiral support. However, we were unable to achieve the stereochemical resolution of these compounds on any achiral HPLC phase.

One possible method to overcome the low efficiency of the BSA-CSP is to reverse the order of the columns. In a coupled chiral–achiral HPLC system, the achiral phase(s) would be used to trap and compress the stereoisomers which were separated on the CSP. The overall effect should be an increase in efficiency and selectivity.

Walhagen and Edholm [3] have described such a coupled chiral–achiral system and successfully used it to separate and quantitate the enantiomers of metoprolol, terbutaline, bupivacaine and oxazepam. The system described by Walhagen and Edholm [3] utilized either an α_1-acid glycoprotein CSP or a BSA-CSP as the initial column and a variety of achiral hydrophobic phases for trapping and separating the resolved enantiomers.

We have utilized and validated a similar system. In our approach, the BSA-CSP was used to separate (6*R*,*S*)-LV and 5-METHF from interfering plasma components and from each other and to stereochemically resolve the diastereomeric (6*S*)- and (6*R*)-LV. The eluent containing (6*S*)-LV was directed onto one C_{18} column and the eluent containing (6*R*)-LV and 5-METHF was directed onto a second C_{18} column. The eluotropic strength of the mobile phase used on the BSA-CSP was not enough to move LV or 5-METHF on the C_{18} column, and the compounds were compressed at the head of the achiral columns. After the columns were loaded, (6*S*)-LV, (6*R*)-LV and 5-METHF were sequentially eluted from the respective columns. The method was validated for plasma levels of each component ranging from 15 to 500 ng/ml and was able to detect LV concentrations of as low as 5 ng/ml.

EXPERIMENTAL

Chemicals

(*R*,*S*)-Leucovorin (folinic acid) and (*R*,*S*)-5-methyltetrahydrofolate were purchased from Sigma (St. Louis, MO, U.S.A.). L-Ascorbic acid and monobasic

sodium phosphate were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The HPLC-grade methanol was purchased from American Scientific (Muskegon, MI, U.S.A.).

Chiral chromatography

The chiral chromatography was performed with a modular liquid chromatograph composed of Beckman 110B solvent delivery module pump (Beckman, Houston, TX, U.S.A.) and a Spectra-Physics SP8780 autosampler equipped with a 100-μl sample loop (Spectra-Physics, San Jose, CA, U.S.A.). The HPLC CSP was composed of BSA bound to 7-μm spherical silica (a 150 mm × 4 mm I.D. Resolvosil BSA-7 column, Alltech Applied Sciences, Deerfield, IL, U.S.A.), and an Adsorbosphere phenyl guard column (Alltech) was placed in front of the column containing the CSP.

The mobile phase was composed of sodium phosphate (0.10 M, pH 5.1), the flow-rate was 1.0 ml/min and the column temperature was maintained at 40 ± 0 1°C with a Flatron Systems CH-30 column heater and TC-50 temperature controller (Rainin Instrument, Woburn, MA, U.S A.).

Elution order of the stereoisomers on the BSA-CSP

The elution order of the diastereomers of LV was previously established as (6*S*), (6*R*) and the elution order of the diastereomers of 5-METHF as (6*R*), (6*S*) [1].

Switching system

The two systems were connected through a ten-port Valco AC10U switching valve equipped with a Rheodyne 7163 pneumatic actuator (Alltech) and a Rheodyne 7000 switching valve equipped with a Rheodyne 7001 pneumatic actuator (Alltech). The switching valve positions used during the chromatography and the function of these positions are presented in Fig. 1. The times presented in Fig. 1 are representative and the acutal times were set at the beginning of each experimental day on the basis of the retention times of (6*S*)-LV, (6*R*)-LV and 5-METHF on the BSA-CSP.

Both switches were controlled by a Spectra-Physics SP4200 computing integrator, and an Autochrom 201 solenoid interface (Alltech) was used to convert the electrical control signals to the corresponding pneumatic control signals.

Achiral chromatography

The achiral chromatography was performed with a Spectra-Physics modular liquid chromatograph composed of a Model SP8800 gradient-capable pumping system, a Spectra-Physics Model 8480 XR UV–Vis detector set at 310 nm, a Shimadzu C-R5A integrator (Shimadzu, Columbia, MD, U.S.A.) and two 125 mm × 4 mm I.D. C$_{18}$ columns (LiChrocart 125-4 RP-18, 5 μm, VWR Scientific, Marietta, GA, U.S.A.).
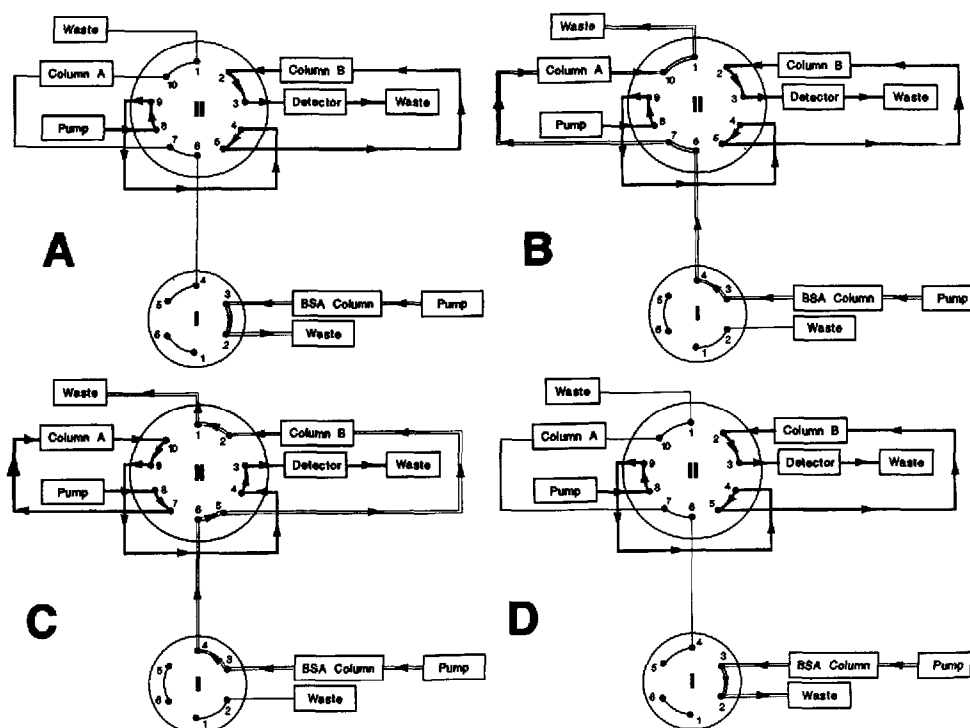
Fig 1 Positions and functions of the switching valves in the coupled column system

Position	Time (min)	Function
A	0 0–16 5	Waste
B	16 5–22.5	Load (6S)-LV on column A
C	22.5–46.5	Load (6R)-LV and 5-METHF on column B, elute (6S)-LV from column A
D	46 5–70.5	Elute (6R)-LV and 5-METHF from column B

The mobile phases used in the achiral chromatography were composed of sodium phosphate (50 mM, pH 5.0)–methanol (100:3.7, v/v) and sodium phosphate (50 mM, pH 5.0)–methanol (90.50, v/v). The gradient mixing program and the related switching valve positions are presented in Table I. During the chromatography, the flow-rate was maintained at 1.0 ml/min and the column temperature was ambient.

Sample preparation

After thawing, plasma samples were centrifuged, and 500 μl were transferred to a 75 mm × 12 mm glass tube Acetonitrile (500 μl) was added, the mixture vortex-mixed and centrifuged for 15 min (2000 g). A fraction of the supernatant

TABLE I

GRADIENT PROGRAM FOR THE ELUTION OF (S)-LEUCOVORIN, (R)-LEUCOVORIN AND 5-METHYLTETRAHYDROFOLATE FROM THE C_{18} COLUMNS

A = sodium phosphate (50 mM, pH 5 0)–methanol (100 3.7, v/v); B = sodium phosphate (50 mM, pH 5 0)–methanol (90 50, v/v). Chromatographic conditions· see text.

Switching valve position[a]	Time (min)	Mobile phase composition	
		A (%)	B (%)
A	0 0–16 5	100	0
B	16.5–22.5	100	0
C	22 5–24 5	100	0
C	24.5–25 5	40	60 (gradient)
C	25 5–27.5	40	60
C	27 5–34 5	5	95 (gradient)
C	34.5–39 5	5	95
C	39.5–44 5	100	0 (gradient)
C	44.5–46.5	100	0
D	46.5–49.5	100	0
D	49 5–53 5	40	60 (gradient)
D	53 5–57 5	40	60
D	57.5–61.5	5	95 (gradient)
D	61.5–66 5	5	95
D	66.5–71 5	100	0 (gradient)

[a] See Fig. 2 for definition of switching valve positions.

(850 μl) was transferred to another 75 mm × 12 mm glass tube and evaporated under reduced pressure in a Speed Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.). The resulting residue was reconstituted in 300 μl of sodium ascorbate solution (200 mg/ml) and transferred to a Centrifree micropartition systems (Amicon, Danvers, MA, U.S.A.). The system was centrifuged for 30 min (2000 g), and 200 μl of the filtrate were transferred to an autosampler vial.

Standard curves

Standard curves were prepared in drug-free plasma at 15, 30, 100, 250 and 500 ng/ml concentrations for each stereoisomer of LV and 5-METHF.

Assay validation

Control samples and samples for within-day and between-day validation studies were prepared using drug-free plasma, and the samples were frozen at −70°C until use. The low calibrators contained 25 ng/ml (6S)-LV, 25 ng/ml (6R)-LV and 50 ng/ml (6R,S)-5-METHF and the high calibrators contained 200 ng/ml (6S)-LV, 200 ng/ml (6R)-LV and 400 ng/ml (6R,S)-5-METHF.

Method application

The suitability of the method to study LV pharmacokinetics was assessed by analyzing plasma samples from a beagle dog that was administered a 25-mg dose of (6*R,S*)-LV by constant-rate infusion over a 20-min period. Heparinized blood samples (4 ml) were collected before and up to 24 h post-dose. Plasma was harvested by centrifugation within 0.5 h of collection. Ascorbic acid (2 mg/ml) was added to plasma as antioxidant prior to storage at −70°C. LV pharmacokinetic parameters were determined by non-compartmental pharmacokinetic methods.

RESULTS AND DISCUSSION

The chromatographic retention, stereoselectivity and resolution on the BSA-CSP of the LV diastereomers and 5-METHF slowly deteriorated during the course of the study. After about 40 injections, the retention times and stereoselectivity for the LV diastereomers and 5-METHF began to slowly decrease and after 300 injections, the columns could no longer be used in the assay. Therefore, the capacity factors (k') of (*S*)-LV, (*R*)-LV and 5-METHF were determined at the beginning of each experimental day and, if necessary, the timing functions and the gradient programs were changed to correspond to the new conditions. A representative chromatogram on the BSA-CSP is presented in Fig. 2. Under the experimental conditions, the k' values of (6*S*)-LV, (6*R*)-LV and (6*S*)-5-METHF were 8 9, 12.4 and 18.2, respectively. For the LV diastereomers, the stereoselectivity (α_s) was 1.39 and the resolution (R_s) was 1.45.

It is of interest to note that (6*R*)-5-METHF coelutes with (6*R*)-LV. This was of no consequence in this study, since *in vivo* (6*S*)-LV is converted into the (6*S*)-5-METHF metabolite while (6*R*)-LV is not converted into the corresponding (6*R*)-
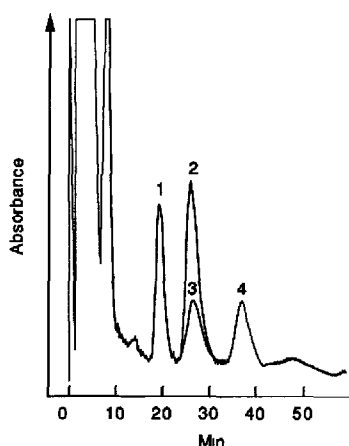

Fig 2 Chromatogram on the BSA-CSP of drug-free plasma spiked with 10 μg/ml (6*S*)-LV, 10 μg/ml (6*R*)-LV and 20 μg/ml (6*R,S*)-5-METHF Peaks: 1 = (6*S*)-LV; 2 = (6*R*)-LV, 3 = (6*R*)-5-METHF; 4 = (6*S*)-5-METHF

metabolite [4,5] and because (6R)-5-METHF and (6R)-LV are ultimately resolved on the C_{18} column (see below). However, if the objective of the study was the measurement of (6R)- and (6S)-5-METHF plasma concentrations following the administration of (6R,S)-5-METHF, this could be accomplished by changing the timing parameters. In this approach, the eluent containing (6R)-5-METHF would be directed to one of the C_{18} columns and the eluent containing (6S)-5-METHF to the other.

The results of the chromatography on the C_{18} columns of a spiked plasma sample are presented in Fig. 3. (6S)-LV is eluted from column A at 37.2 min and (6R)-LV and 5-METHF are eluted from column B at 64.1 and 66.2 min, respectively. The resolution factor between (6R)-LV and 5-METHF was 4.5. Since the individual 5-METHF isomers were not available, the plasma was spiked with the commercially available mixture of the diastereomers. As stated above, this will present no problem in the analysis of biological samples.

Standard curves were constructed by plotting the (6S)-LV, (6R)-LV and 5-METHF peak areas *versus* the spiked plasma concentrations. Since each respective solute was quantitatively transferred from the BSA-CSP to the C_{18} columns, no internal standard was necessary. The resulting curves were linear over the range investigated and were expressed by the following equations: (6S)-LV: $y = 298x + 16047$, $r^2 = 0.998$; (6R)-LV: $y = 309x + 10124$, $r^2 = 0.999$; 5-METHF: $y = 327x + 16112$, $r^2 = 0.995$.

The results of the determination of the precision and reproducibility of the method are presented in Table II. In all cases, accuracy was within 5% of theoretical, and precision, expressed as coefficient of variation (C.V.), was less than 5%.
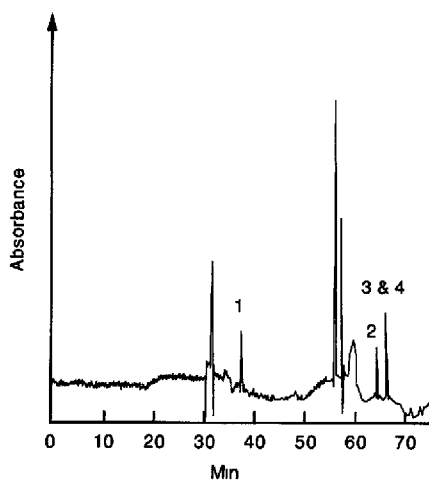

Fig. 3. Chromatogram on the C_{18} column of drug-free plasma spiked with 30 ng/ml (6S)-LV, 30 ng/ml (6R)-LV and 60 ng/ml (6R,S)-5-METHF Peaks: 1 = (6S)-LV; 2 = (6R)-LV, 3 + 4 = (6R,S)-5-METHF From 0 to 30 min no elution from column A or B, from 30 to 58 min, elution from column A, from 58 to 82 min elution from column B.

TABLE II

ASSAY VALIDATION

See text for experimental conditions.

Compound	Actual concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	C.V. (%)
Within-day (n = 5)				
(6S)-LV	25 0	25.2 ± 1.1	100 4	4.4
(6R)-LV	25.0	25 8 ± 0 8	103 2	3.2
5-METHF	50 0	49 8 ± 1 6	99.8	3 3
(6S)-LV	200 0	197.3 ± 3.6	98 6	1 8
(6R)-LV	200 0	199.4 ± 8 8	99 8	4 4
5-METHF	400.0	399.8 ± 6 4	99 8	1.6
Between-day (n = 15)				
(6S)-LV	25.0	25 7 ± 1 2	102.8	4.7
(6R)-LV	25.0	25.9 ± 0.9	103 6	3 4
5-METHF	50.0	47 9 ± 1 7	95 7	3 7
(6S)-LV	200.0	196 8 ± 7 1	98.4	3.6
(6R)-LV	200.0	199.1 ± 7.5	99 5	3 8
5-METHF	400.0	398 9 ± 13 0	99 7	3 3

Representative chromatograms from blank canine plasma and canine plasma obtained 1 h after the administration of 25 mg of (6R,S)-LV by intravenous route to a beagle dog are presented in Fig. 4A and B, respectively. The plasma concentration *versus* time curve following the intravenous administration is presented in Fig. 5. Peak (6S)-LV and (6R)-LV concentrations of 4.8 and 5.6 μg/ml, respectively, were reached at the end of the 20-min infusion. Thereafter, LV concentra-
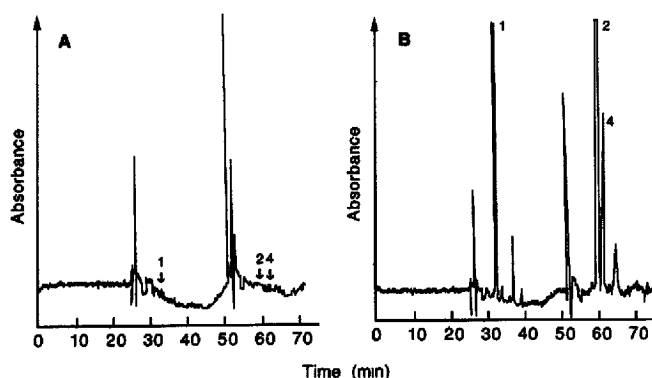

Fig. 4 Analysis of plasma samples on the coupled-column system. (A) Blank plasma, (B) sample taken 1 h after intravenous administration. For peak identification, see Fig. 2
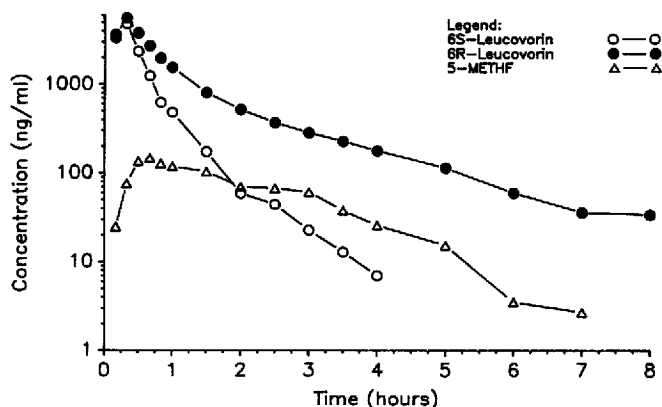
Fig. 5. Plasma concentration–time profiles of folates after administration of a 25-mg intravenous dose of (R,S)-LV to a beagle dog.

tions declined in at least a biphasic manner. (6S)-LV and (6R)-LV terminal half-life, systematic clearance and steady state volume of distribution values were 0.6 and 1.5 h, 3.8 and 7.6 ml/min/kg and 269 and 165 ml/kg, respectively. The peak 5-METHF concentration of 147 ng/ml was reached 40 min post-dose. (6S)-LV, (6R)-LV and 5-METHF could be quantitated up to 4, 6 and 7 h, respectively, post-dose.

CONCLUSION

One of the major problems associated with the application of many HPLC CSPs to bioanalytical work is their low efficiency which hinders the detection of sub-microgram quantities. This is especially true for biopolymer-based CSPs. The method described in this paper is one approach to overcoming this difficulty. It takes advantage of the difference in elutropic strengths between the mobile phase on the BSA-CSP and the achiral C_{18} columns to compress the broad peaks from the chiral phase into sharp spikes. The result was an over 100-fold increase in sensitivity. In addition, while the time for a single experimental run is over 70 min, the system is constructed so that all three columns are at different stages in the analytical process at the same time and the assays can be overlapped.

ACKNOWLEDGEMENTS

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