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Note

Direct resolution of the stereoisomers of leucovorin and 5-methyltetrahydrofolate using a bovine serum albumin high-performance liquid chromatographic chiral stationary phase coupled to an achiral phenyl column

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Leucovorin (LV) is a reduced folate which is used to treat or prevent host toxicity in cancer patients due to the administration of supralethal doses of methotrexate [1]. High doses of LV have also been used in conjunction with 5-fluorouracil (5-FU) and this combination has produced better results in adult patients with advanced colorectal cancer than the administration of 5-FU alone [2].

LV is administered as a diastereomeric mixture of the 5-formyl derivative of tetrahydrofolic acid (Fig. 1A). The diastereomers differ in the chirality at the 6-carbon of the tetrahydropteridine ring while the chirality of the *l*-glutamic acid moiety is fixed in both forms of the molecule. Initial studies [3] have indicated that the (6*S*)-isomer (*l*-LV) is the biologically active isomer and that after administration (6*S*)-LV is rapidly converted to an active metabolite, 5-methyltetrahydrofolate (5-METHF) (Fig. 1B) [4]. In addition, the plasma half-lives of (6*S*)-LV and 5-METHF are significantly shorter than that of the (6*R*)-isomer of leucovorin (*d*-LV), 32, 224 and 485 min, respectively [4]. The effect of the inactive (*R*)-LV on the metabolism, disposition and clinical efficacy of the (*S*)-isomer is unknown nor has the isomeric composition of the 5-METHF been established.

The current methods for the determination of the concentrations of (*S*)- and (*R*)-LV and 5-METHF are based on biochemical assays of intact serum samples or of eluent fractions from a high-performance liquid chromatographic (HPLC) separation [4,5]. Hamel et al. [5] have described an assay for LV which is based on the formylation of methionyl-tRNA in a cell-free extract derived from *Esch-*

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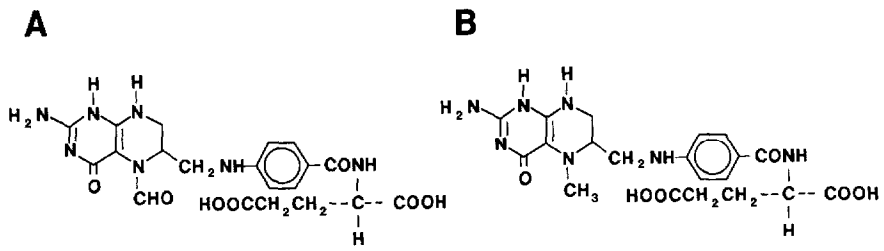

Fig. 1. Structures of (A) leucovorin and (B) 5-methyltetrahydrofolate.

erichia coli. The method is specific for (*S*)-LV and 5-METHF but does not quantity (*R*)-LV. Straw et al. [4] have utilized an assay which involves an initial HPLC separation followed by assay (the growth of *Lactobacillus casei*) of the fractions to determine the (*S*)-LV concentration.

In order to facilitate the analysis of the stereoisomeric composition of LV and 5-METHF in serum after the clinical administration of LV, we have developed a direct HPLC analysis for the diastereoisomers of LV and 5-METHF. The compounds are resolved on a commercially available HPLC chiral stationary phase based on immobilized bovine serum albumin (BSA-CSP). This system was adapted for the analysis of serum samples by coupling the BSA-CSP to an achiral column packed with a phenyl support. The LV and 5-METHF fractions can be isolated from interfering serum peaks and quantified using the phenyl column and then selectively switching to the BSA-CSP for the determination of the stereoisomeric composition.

EXPERIMENTAL

Apparatus

The achiral chromatography was performed with a Spectra-Physics (Santa Clara, CA, U.S.A.) modular liquid chromatograph composed of SP Model 8700 gradient capable pumping system, SP Model 8773XR UV–Vis detector set at 310 nm (0.025 a.u.f.s.), SP Model 4270 computing integrator, Rheodyne 7125 injection valve (Rainin Instrument, Woburn, MA, U.S.A.) and μBondapak Phenyl HPLC column (10 μm, 300×3.9 mm I.D., Waters Chromatography Division, Milford, MA, U.S.A.)

The chiral chromatography was performed with a Spectra-Physics modular liquid chromatograph composed of SP Model 8770 pumping system, SP Model 877XR UV–Vis detector set at 310 nm (0.010 a.u.f.s.), SP Model 4200 computing integrator HPLC column composed of bovine serum albumin bound to 10-μm spherical silica (Resolvosil, Rainin Instrument). The column temperature was maintained within ±0.1°C of the desired setting with a Flatron Systems CH-30 column heater and TC-50 temperature controller (Rainin Instrument).

The two systems were connected through a Rheodyne 7000 switching valve equipped with a pneumatic actuator and a 1-ml sample loop (Rainin Instrument).

Chemicals

(*R,S*)-Leucovorin (folinic acid), (*R,S*)-5-methyltetrahydrofolate and methotrexate [(+)-amethopterin] were purchased from Sigma (St. Louis, MO, U.S.A.). (*S*)-LV and (*R*)-5-METHF were kindly supplied by Dr. Youcef Rustum (Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY, 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.).

Chromatographic conditions

The mobile phases used in this study were composed of 0.25 *M* phosphate buffer, pH 5.0 (mobile phase A) and 0.25 *M* phosphate buffer, pH 5.0–methanol (1:1, v/v) (mobile phase B).

The achiral chromatography was carried out using the following gradient elution program: 0–15 min, 100% mobile phase A; 15–30 min, 100% mobile phase A to mobile phase A–B (50:50); 30–35 min, mobile phase A–B (50:50) to 100% mobile phase B; 35–40 min 100% mobile phase B. The chromatography was performed at ambient temperature with a flow-rate of 2 ml/min.

The chiral chromatography was carried out using mobile phase A, a column temperature of 40°C and a flow-rate of 0.5 ml/min.

Sample preparation

Standard solutions of LV and 5-METHF were prepared using an aqueous solution of ascorbic acid (1 mg ascorbic acid per ml water).

Blood samples from both patients and normal volunteers were collected in heparinized tubes to which ascorbic acid (2 mg/ml of blood) had been added. The tubes were centrifuged in the cold at 800 *g* and the plasma stored at −15°C until analyzed.

After thawing, 10 μl of the internal standard solution (methotrexate, 30 μg/ml in an aqueous solution of 1 mg/ml ascorbic acid) were added followed by the addition of 1.5 ml of methanol. The mixture was vortexed and centrifuged at 800 *g* and the supernatant collected. The supernatant was dried under a stream of nitrogen. The residue was reconstituted in 200 μl of water and injected onto the chromatographic system.

Standard curves

Standard curves were constructed for (*R*)- and (*S*)-LV and (*R*)- and (*S*)-5-METHF on the BSA-CSP using aqueous standards. The on-column concentration ranged from 35 to 560 ng and the minimum detectable concentration was 6.5 ng for each isomer. The curve for each compound was linear over the concentration range. The (*S*)-LV standard curve had a calculated standard deviation of 0.042 and correlation coefficient of 0.996, the (*R*)-LV curve 0.058 and 0.996, the (*S*)-5-METHF curve 0.116 and 0.988, and the (*R*)-5-METHF curve 0.043 and 0.998.

Standard curves for LV and 5-METHF on the achiral column were prepared using spiked serum samples. The concentration of LV ranged from 2500 to 78

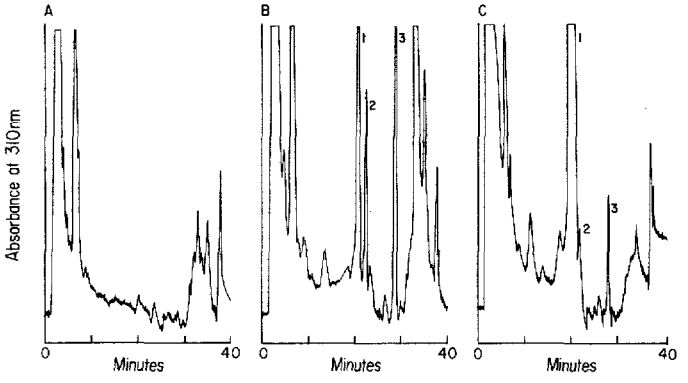

Fig. 2. Representative chromatograms on the achiral phenyl support of extracted blank serum (A), serum with added leucovorin (12 500 ng/ml), 5-methyltetrahydrofolate (1250 ng/ml) and internal standard (300 ng/ml) (B) and serum sample from a patient 15 min post-infusion (C). See Experimental for chromatographic conditions. Peaks: 1 = leucovorin; 2 = 5-methyltetrahydrofolate; 3 = internal standard.

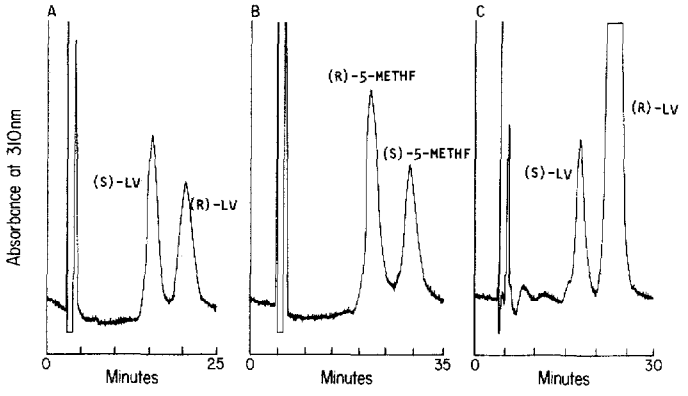

Fig. 3. Representative chromatograms on the BSA-CSP of a leucovorin serum standard (419 ng/ml per isomer) after switching from the achiral system (A), a 5-methyltetrahydrofolate serum standard (419 ng/ml per isomer) after switching from the achiral system (B) and leucovorin from a patient serum sample acquired 4.5 h post infusion (C). See Experimental section for chromatographic conditions.

ng/ml and the concentration of 5-METHF ranged from 250 to 8 ng/ml. The LV standard curve had a calculated standard deviation of 3.451 and a correlation coefficient of 0.995. The 5-METHF standard curve had a calculated standard deviation of 1.689 and a correlation coefficient of 0.986.

RESULTS AND DISCUSSION

The results from the chromatography on the achiral phenyl column are illustrated in Fig. 2. Under the chromatographic conditions used in this study, the capacity factors (k') for LV, 5-METHF and methotrexate were 13.5, 14.9 and 19.7, respectively.

The results from the chromatography on the BSA-CSP of LV and 5-METHF

TABLE I

SERUM CONCENTRATION OF *S*- AND *R*-LEUCOVORIN (LV) AND *S*- AND *R*-5-METHYLTETRAHYDROFOLATE (5-METHF) AFTER A 4-h INFUSION OF 528 mg LV

Time post-infusion (h)	Concentration (ng/ml)			
	(*S*)-LV	(*R*)-LV	(*S*)-5-METHF	(*R*)-METHF
4.5	77.34	589.38	27.84	0.00
9	0.00	426.25	17.69	0.00
18	0.00	52.55	1.75	0.00

after preseparation on the achiral system are illustrated in Fig. 3. Under the chromatographic conditions used in this study, the k' values for (*S*)-LV and (*R*)-LV were 2.76 and 3.97, respectively, and the stereochemical resolution (α) was 1.44. The k' values for (*R*)-5-METHF and (*S*)-5-METHF were 2.88 and 4.52, respectively, and α was 1.57.

The coupled column system was applied to serum samples from a patient who had received 528 mg of LV in a 4-h infusion. The results are presented in Table I and are consistent with previously reported studies using normal volunteers [4].

CONCLUSION

The coupled achiral–chiral chromatographic system described in this paper is capable of determining the serum concentrations of the stereoisomers of LV and 5-METHF after the clinical administration of LV. The applicability of this method to pharmacokinetic studies of LV and 5-METHF and its usefulness in other pharmacological studies will be reported elsewhere.

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REFERENCES

1 J.R. Bertino, Cancer Treat. Rep., 65 (Suppl. 1) (1981) 131.
2 G. Tisman, V. Flener, M.E. Jones and L. Buck, Proc. Ann. Meet. Am. Assoc. Cancer Res., 26 (1985) 159.
3 B. Mehta, A.L. Gisolfi, D.J. Hutchison, A. Nirenberg, M.G. Kellick and G. Rosen, Cancer Treat. Rep., 62 (1978) 345.
4 J.A. Straw, D. Szapary and W.T. Wynn, Cancer Res., 44 (1984) 3114.
5 E. Hamel, G. Johnson and D. Blaubiger, Cancer Treat. Rep., 65 (1981) 545.