Journal of Chromatography, 487 (1989) 456–462
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4533

Note

Determination of reduced folate derivatives in tissue samples by high-performance liquid chromatography with fluorimetric detection

GARY L. CASE and ROBERT D STEELE*

Department of Nutritional Sciences, University of Wisconsin-Madison, 1415 Linden Drive, Madison, WI 53706 (U.S.A.)

(First received August 16th, 1988; revised manuscript received October 6th, 1988)

The procedure developed by Gregory et al. [1] for the extraction and quantitation by high-performance liquid chromatography (HPLC) of reduced folate compounds is appealing because it is relatively rapid and the major endogenous folates are resolved into three basic monoglutamate derivatives, tetrahydrofolate (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), and 5-formyltetrahydrofolate (5-HCO-H_4folate). In addition, detection is based upon the native fluorescence of these derivates and is therefore highly sensitive. An inherently restrictive feature of the method is the low fluorescence intensity of 5-HCO-H_4folate as compared to the other two derivatives. Obtaining a quantifiable peak for this derivative in tissue extracts is frequently difficult, requiring that the HPLC column be loaded with a large sample volume and that a highly sensitive spectrofluorimeter be used for detection [1]. This report describes modifications of the original procedure that result in less dilution of the extracted folates during the work-up and in an improved elution profile from the reversed-phase HPLC column, both of which aid in accurately determining the 5-HCO-H_4folate derived from tissue samples.

EXPERIMENTAL

Sample extraction

Samples of freshly obtained rat liver (approximately 2 g) were weighed into 50-ml polycarbonate centrifuge tubes and 4 volumes of 0.1 *M* sodium acetate, pH 4.9–0.5% ascorbate–20 m*M* 2-mercaptoethanol were added. Homogenates were prepared using a Tissuemizer (Tekmar, Cincinnati, OH, U.S.A.) under a flowing

0378-4347/89/$03.50

stream of nitrogen to reduce oxygenation of the homogenate. Approximately 8 ml of the homogenate were transferred to 16 mm × 75 mm screw-cap polycarbonate ultracentrifuge tubes, the head space was flushed with nitrogen, and the tubes were sealed and heated in a boiling water bath for 60 min. After cooling on ice for about 15 min, the samples were centrifuged at 20 000 *g* for 15 min.

Conjugase treatment

The supernatant from the heat-treated sample (5 ml) was transferred to the outer shell of a Centriprep-30 ultrafiltration device (Amicon, Danvers, MA, U.S.A.), 0.3 ml of crude hog kidney conjugase, prepared as described previously [1], was added, and the tubes were flushed with nitrogen, capped and incubated for 60 min in a 37°C water bath. The center membrane filter holder was then inserted and 4–4.5 ml of ultrafiltrate from the incubation suspension were obtained by centrifuging the filter device two or three times for 6 min at 1500 *g* in a clinical centrifuge.

Preparative chromatography on DEAE-Sephadex

Interfering non-folate fluorescent compounds were removed by chromatography over a miniature column of DEAE-Sephadex A-25 (Pharmacia, Piscataway, NJ, U.S.A.). A 4-ml volume of the sample utlrafiltrate was passed through a 3.0 cm × 0.5 cm column of gel that had been equilibrated with 0.1 *M* sodium acetate, pH 4.9. The flow-rate was set at 0.4 ml/min with a peristaltic pump (Minipuls 2, Gilson Medical Electronics, Middleton, WI, U.S.A.) connected to the column outlet tubing. The column was then rinsed with 4 ml of 0.1 *M* sodium acetate, pH 4.9–1% ascorbate. For routine analyses, all effluent up to this point was discarded. Finally, the column was eluted with 5 ml of 0.2 *M* sodium acetate, pH 4.9–1.0 *M* sodium sulfate–1% ascorbate at a reduced flow-rate of 0.2 ml/min. The resulting 5 ml of effluent were collected for chromatography by HPLC. Normally, four samples were chromatographed simultaneously using a single four-channel pump head. Samples that could not be analyzed immediately by HPLC were flushed with nitrogen and stored in sealed glass vials at −80°C. The extracted folates were stable for at least two weeks when stored under these conditions.

High-performance liquid chromatography

Reversed-phase HPLC was performed using a Waters (Milford, MA, U.S.A.) system consisting of an automatic gradient controller, two pumps and either a 300 mm × 3.9 mm stainless-steel column or a 100 mm × 10 mm Radial-Pak column, both filled with the μBondapak phenyl packing (Waters). The system was equipped with a pre-column as described [1], but no guard column was used. Peak detection was with a McPherson (Acton, MA, U.S.A.) Model FL-750 spectrofluorimeter equipped with a 6-μl flow cell and adjusted for an excitation wavelength of 300 nm and an emission wavelength of 356 nm. Two mobile phases containing either 5 or 15% acetonitrile in 0.033 *M* sodium dihydrogenphosphate, pH 2.3, were used. The column was equilibrated with the 5% acetonitrile mobile phase at 2.0 ml/min. Following sample injection, the column was eluted isocrat-

ically with the initial mobile phase for 6 min, followed by a rapid, hyperbolic gradient change to a 25:75 mixture of the 5:15% acetonitrile-containing buffers over a 2-min period. Elution continued isocratically with the resulting 12.5% acetonitrile mobile phase until 12 min after injection, at which time another hyperbolic gradient of 2 min duration was used to return to the starting condition (5% acetonitrile) for re-equilibration. Using this sequence, samples could be injected onto the column at 20-min intervals. For the present study, quantitation was based upon measuring peak height in comparison to that produced by chromatographing standard solutions of the folate derivatives. Peak height is directly proportional to the concentration of each derivative, providing that the elution time remains relatively constant from analysis to analysis.

Chemicals and other special materials

Tetrahydrofolic acid, DL-5-methyltetrahydrofolic acid (barium salt) and 5-formyltetrahydrofolic acid (calcium salt) used to prepare standard solutions were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile was of HPLC grade. All other chemicals were of reagent grade. All buffers used in the reduced folates extraction and analysis procedures were freshly prepared and degassed by vacuum ultrafiltration on the day of use.

RESULTS AND DISCUSSION

The results presented are those directly related to our modifications of the procedure initially described by Gregory et al. [1].

Centrifugal ultrafiltration following conjugase treatment

Using the Centriprep-30 ultrafiltration devices for the conjugase treatment step has no direct effect on either sample dilution or recovery. We found this modification to be beneficial, however, because it eliminated the occasional plugging of the gel columns with particulate matter introduced from the unfiltered supernatant/crude hog kidney conjugase suspension. Routine use of the ultrafiltration step also reduced problems with excessive pressure build-up and loss of resolution of the HPLC column.

Clean-up chromatography on DEAE-Sephadex A-25

In the original method, interfering compounds were removed by adjusting the conjugase-treated tissue extract to pH 7.0, followed by chromatography on a 10 cm $\times$ 0.7 cm column of DEAE-Sephadex A-25. Following extensive rinsing, the column was eluted with 70 ml of a pH 7.0 phosphate buffer containing 1 *M* sodium sulfate. The reduced folate derivatives were recovered in the last 50 ml of the effluent. We observed that the folate derivatives were bound to the top few millimeters of gel in this larger column following the sample load and rinse steps. They could be recovered quantitatively by transferring the top 1 cm of gel to a smaller column, followed by elution with approximately 5 ml of the high salt buffer. Although this method was effective in achieving a more highly concentrated sample for HPLC analysis, a less tedious approach was developed in which

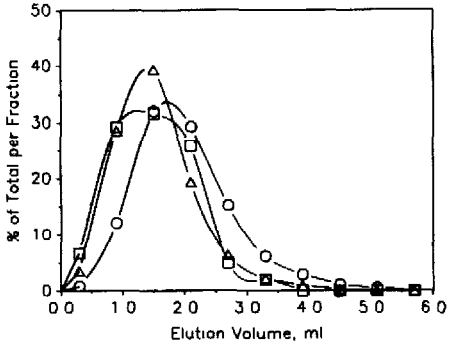

Fig. 1. Elution of reduced folate monoglutamate derivatives from the miniature column of DEAE-Sephadex A-25. A 4-ml volume of conjugase-treated extract from rat liver homogenized in buffer containing added folates standards (4 nmol/ml each) was loaded onto the 3.0 cm×0.5 cm column and rinsed with 4 ml of 0.1 *M* sodium acetate, pH 4.9–1% ascorbate. The column was then eluted with 0.2 *M* sodium acetate, pH 4.9–1.0 *M* sodium sulfate–1% ascorbate at a flow-rate of 0.2 ml/min. Fractions of the effluent (0.6 ml) were collected and analyzed for reduced folates by HPLC. The concentrations of H_4folate (○), 5-CH_3-H_4folate (△) and 5-HCO-H_4folate (□) in each fraction are expressed as the percentage of the total amount of each derivative recovered in all fractions.

the preparative chromatography was carried out over a small (3.0 cm×0.5 cm) DEAE-Sephadex A-25 column at pH 4.9 as described under Experimental. The use of the lower-pH buffer system eliminates the need for adjusting the sample pH and also decreases retention of the principal fluorescent contaminants by the column during the load and rinse steps.

The elution of reduced folate monoglutamates from the 3.0 cm×0.5 cm clean-up column is shown in Fig. 1. In this experiment, 4 ml of conjugase-treated extract from rat liver (representing approximately 0.8 g of liver) were passed through the column, followed by 4 ml of acetate–ascorbate buffer rinse. The column was eluted with the acetate–sulfate–ascorbate buffer and 0.6-ml fractions of effluent were collected for assay by HPLC as described in Experimental. H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate were almost completely eluted in a 4-ml volume and no detectable folate peaks were observed in fractions beyond 5 ml. A regulated flow-rate is essential in order to obtain reproducible elution of the folates from the DEAE-Sephadex column. Flow-rates greater than 0.2 ml/min during elution resulted in significant tailing, particularly of the 5-HCO-H_4folate, so that a 5-ml cut-off did not result in quantitative sample recovery.

Sequential isocratic–gradient elution of the reversed-phase HPLC column

The original method [1] used a linear mobile phase buffer gradient (7.5–11.3% acetonitrile) to elute the folate derivatives from the reversed-phase phenyl column. Fell and Steele [2] achieved comparable results using isocratic elution with 7.5% acetonitrile in the mobile phase. With either of these approaches, the 5-formyl derivative elutes last as a relatively broad peak dispersed in 5–6 ml of mobile phase. The modified elution buffer scheme described under Experimental was developed in order to elute 5-HCO-H_4folate from the column earlier as a narrow band, while maintaining the separation between the closely eluting

H_4folate and 5-CH_3-H_4folate derivatives. A chromatogram showing the elution of reduced folates from a 100-μl sample of rat liver extract is shown in Fig. 2C. The peaks shown as dotted lines overdrawn on this chromatogram are from a second sample prepared in parallel from the same rat liver, except internal standards of the three reduced folate compounds were added to the homogenizing buffer. The mobile phase gradient profile, expressed as percentage acetonitrile, is indicated by a dashed line. The H_4folate and 5-CH_3-H_4folate peaks are well separated and 5-HCO-H_4folate, in comparison to previously described results [1,2], is eluted several minutes earlier as a narrower peak. Fig. 2A and B illustrate the effectiveness of the Sephadex preparatory chromatography carried out as described above. The chromatogram in Fig. 2A represents a 100-μl sample of conjugase-treated liver extract before preparatory chromatography. Fig. 2B shows the chromatogram produced by injecting 200 μl of the eluate collected from the Sephadex column after passing 4 ml of the same liver extract and 4 ml of buffer rinse over it. None of these fluorescent compounds, which are removed during the preliminary chromatography, correspond to the elution positions of the reduced folate derivatives (Fig. 2C, dotted lines).

An experiment was undertaken to measure the recovery of reduced folates added to tissue samples. Liver samples were obtained from six 500–600 g male rats of the Sprague–Dawley strain that were used as control animals in an unrelated

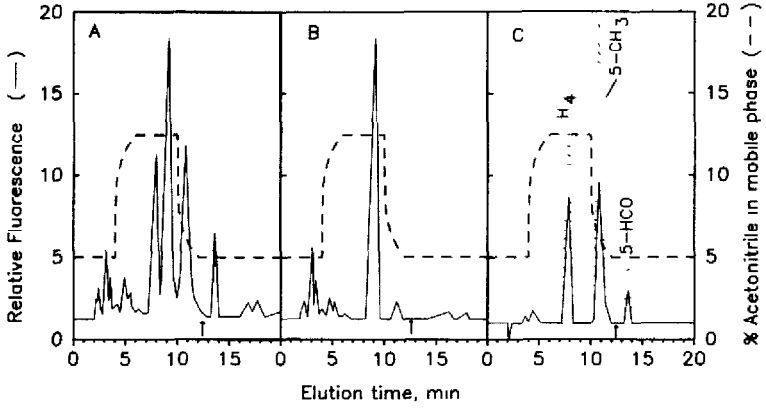

Fig. 2 HPLC profiles of a conjugase-treated extract from rat liver. A sample of rat liver was homogenized, extracted, and treated with conjugase as described under Experimental. (A) The injected sample was 100 μl (representing about 80 mg of liver) of extracted sample that was not subjected to preliminary chromatography over DEAE-Sephadex A-25. (B) The injected sample was 200 μl of the pooled 8 ml of the effluent from the DEAE-Sephadex column with 4 ml of the rat liver extract and rinsing with 4 ml of 0.1 *M* sodium acetate, pH 4.9–1% ascorbate. (C) The injected sample was 100 μl of the folate-containing fraction eluted from the DEAE-Sephadex column with 5 ml of 0.2 *M* sodium acetate, pH 4.9–1.0 *M* sodium sulfate–0.5% ascorbate. Peaks shown by dotted lines are overdrawn from a separate chromatogram of a duplicate liver sample prepared in buffer containing added internal folate standards [4 nmol/ml each of H_4folate (H_4), 5-CH_3-H_4folate (5-CH_3), and 5-HCO-H_4folate (5-HCO)]. The dotted line on each chromatogram indicates the sequential isocratic–hyperbolic mobile phase gradient (as percentage acetonitrile) used in elution of the reversed-phase phenyl column. The arrow indicates the time at which the detector sensitivity was increased from 0.1× to 0.03× for detection of the 5-HCO-H_4folate peak.

TABLE I

EFFECT OF TYPE OF DIET ON THE CONCENTRATIONS AND RELATIVE DISTRIBUTIONS OF REDUCED FOLATES IN RAT LIVER

Each value is the mean ± S.D. of three observations.

Diet[a]	H_4folate		5-CH_3-H_4folate		5-HCO-H_4folate		Total (nmol/g)
	nmol/g	%	nmol/g	%	nmol/g	%	
Commercial	6.3 ± 2.8	66.3 ± 1.7	4.0 ± 0.9	16.7 ± 5.7	4.3 ± 2.2	17.3 ± 8.7	24.3 ± 3 1
Casein	10.2 ± 2.8	46.7 ± 4 0	7.5 ± 0.7	35.3 ± 4.0	3 8 ± 0.7	17.8 ± 0.7	21.6 ± 4.2
p^b	< 0.10	< 0.01	< 0.01	< 0.025	N.S.	N.S.	N.S.

[a]Rats were fed either a standard commercial ration or a purified casein-based diet in pelleted form for four weeks prior to measuring liver folates.
[b]Probability level of difference between mean values in each column using a *t*-test. Levels greater than 0.10 were considered to be non-significant (N.S.).

experiment. Three rats had been fed a purified diet based on 18% casein [3] for four weeks, while the other three had been fed a standard commercial diet (Wayne Rodent Blox). Duplicate samples of each liver were prepared for folate analysis using the modified procedure, except that one sample from each was homogenized in buffer supplemented with 4 nmol/ml of each reduced folate derivative. Mean (± S.D.) recoveries of added folates were 82.2 ± 2.8% for H_4folate, 92.0 ± 16.0% for 5-CH_3-H_4folate and 79.8 ± 18.0% for 5-HCO-H_4folate. These recovery values are similar to those reported for the original procedure [1].

The results of this recovery experiment also indicated that the relative distribution of liver folates differed between rats fed the commercial ration and purified diets. As shown in Table I, livers of rats fed the commercial ration had a significantly greater proportion of H_4folate (66 versus 47%) and significantly less 5-CH_3-H_4folate (17 versus 35%) in comparison with livers of rats fed the casein-based diet. The total folate concentration and the proportion of 5-HCO-H_4folate did not differ significantly. Reported differences in the relative distribution of rat liver folates have been used as a basis to compare several different HPLC-based analytical methods [4]. Our results, however, suggest that nutritional factors as well as analytical methodology may contribute to the variability in endogenous rat liver folates that have been reported by different investigators.

In summary, the modifications reported here improve the original method by yielding a final sample extract that is concentrated in a 5-ml instead of a 50-ml volume and by improving the relative sensitivity for fluorimetric detection of 5-HCO-H_4folate through manipulation of the mobile phase elution gradient. These improvements permit the quantitative measurement of reduced folates extracted from less than 1 g of liver and allow the use of a conventional flow-cell spectrofluorimeter rather than a more expensive high-sensitivity instrument for peak detection.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grant DK35853 and the College of Agricultural and Life Sciences.

REFERENCES

1 J.F. Gregory, D.B. Sartain and B.D.F. Day, J. Nutr., 114 (1984) 341.
2 D. Fell and R.D. Steele, Arch Biochem. Biophys., 240 (1985) 843.
3 A.A. Jerkins and R.D. Steele, Hepatology, 8 (1988) 855.
4 S.D Wilson and D.W. Horne, Anal. Biochem., 142 (1984) 529