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Note

Microdetermination of folate monoglutamates in serum by liquid chromatography with electrochemical detection

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The naturally occurring derivatives of pteroylglutamic acid (PteGlu) function as essential coenzymes in the biosynthesis of protein and nucleic acids [1]. The folate compounds differ in the state of oxidation of pteridine ring, in the substitution at the 5- and/or 10-nitrogen positions and in the number of glutamic acid residues [1]. Although the folate compounds in mammalian tissues exist primarily as polyglutamates [2], the monoglutamates are present in body fluids such as plasma and bile [3]. Analysis of the polyglutamates is usually performed after hydrolysis to the corresponding monoglutamates. Several high-performance liquid chromatographic (HPLC) methods have been proposed for the separation of the monoglutamates, based on anion-exchange, ion-pair and reversed-phase chromatography [4—12]. Although microbiological assay [13] and fluorescence methods [14] are as sensitive as the HPLC methods, the former is time-consuming and the latter has poor reliability [15]. Recently, the concentrations of exogenous 5-formyltetrahydrofolic acid (5-formyl-H_4PteGlu) [16] and of 5-methyl-H_4PteGlu in plasma have been determined by electrochemical detection (ED) using HPLC [8]. However, only the

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single specific folate form could be analysed with each assay procedure, and a substantial amount of sample serum was required. 5-Methyl-H_4PteGlu is a major folate derivative in blood [17]. It ranges from 3 to 16 ng/ml in normal human serum [8, 18], and above 30 ng/ml in rat serum [19], as determined by HPLC—ED [8] and by radioassay [18, 19].

This paper describes an HPLC—ED assay suitable for the sensitive and selective analysis of various folate monoglutamates. This assay was applied to the microdetermination of an endogenous 5-methyl-H_4PteGlu in human and rat sera.

EXPERIMENTAL

Liquid chromatography

HPLC was performed using a JASCO (Japan Spectroscopic Co., Tokyo, Japan) TRI ROTAR-V system with a VL-614 variable-loop injector, an Irica guard filter (10/0.45/10 μm; Irica Instruments, Kyoto, Japan), a Cosmosil 5Ph (a 5-μm phenyl bonded phase) column (Nakarai Chemicals, Kyoto, Japan), 150 × 4.6 mm I.D., an Irica E-502 amperometric detector with a glassy carbon electrode and a JASCO DP L-220 data processor. The mobile phase was a solution of 50 m*M* potassium dihydrogen phosphate (pH 3.5) containing 0.1 m*M* disodium EDTA and 15% methanol. The flow-rate was 0.5 ml/min.

Preparation of serum sample

Blood samples, taken before breakfast from five healthy human volunteers between the ages of 22 and 32 years, and from two fasted male Sprague—Dawley (257 g, 300 g) and three fasted male Wister rats (243 ± 18 g) were collected via the inferior cava after being anesthetized with 55 mg/kg sodium pentobarbital. Serum was prepared from clotted blood. To the serum, 2-mercaptoethanol was added to a final concentration of 0.1 *M*, and the sample was frozen at —30°C until use. The serum (200 μl) was mixed with 2 vols. of acetone, and the precipitate was removed by centrifugation at 13 000 *g* for 2 min. The supernatant was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and 30 μl of the sample were used for the HPLC analyses.

Standard compounds

PteGlu, H_4PteGlu, 5-methyl-H_4PteGlu sodium salt and 5-formyl-H_4PteGlu calcium salt were purchased from Sigma (St. Louis, MO, U.S.A.). 7,8-Dihydrofolic acid was prepared by the dithionite method of Futterman [20] as modified by Blakley [21]. 10-Formyl-H_4PteGlu and 5,10-methylidyne-H_4PteGlu were synthesized by the method of Scott [22]. Standard solutions were prepared by dissolving each compound in 0.2 *M* 2-mercaptoethanol containing 0.1% sodium ascorbate to protect it from oxidative decomposition. They were frozen at —30°C and protected from light until use.

RESULTS AND DISCUSSION

Electrochemistry of folate monoglutamates

In order to determine folate monoglutamates electrochemically, the electro-

chemical behaviour of the compounds was studied by repeated injections of a standard solution and by detecting at different potentials. Fig. 1 shows the voltammograms for five derivatives of PteGlu obtained under the protocol conditions. The information is used in establishing the optimum operating potential of the detector for the detection of the folate derivatives. Although the potential of +750 mV versus Ag/AgCl reference electrode is suitable for the detection of the standard compounds, 5-methyl-H_4PteGlu in serum was decreased at +350 mV. This potential was chosen as a compromise between the maximal current response and minimal background current from other oxidizable components in serum.

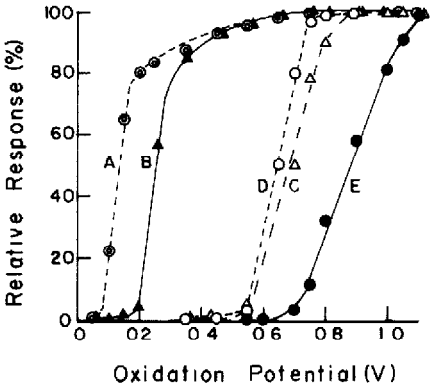

Fig. 1. Voltammograms for folate monoglutamates. (A) H_4PteGlu; (B) 5-methyl-H_4PteGlu; (C) 5-formyl-H_4PteGlu; (D) 10-formyl-H_4PteGlu; (E) PteGlu. Average of four separate experiments. The standard error of the mean did not exceed ±10%.

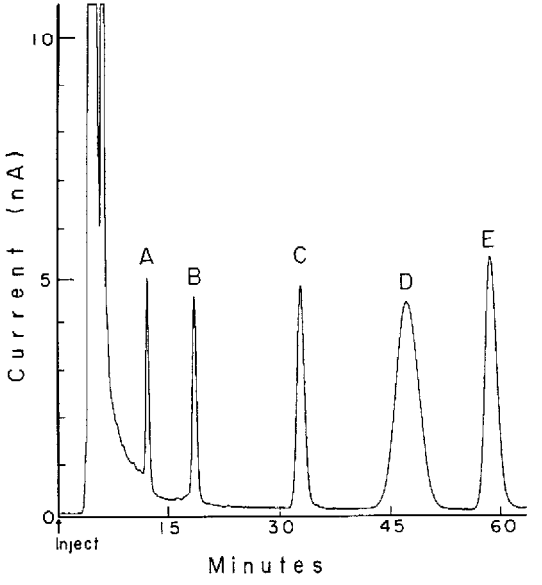

Fig. 2. Chromatogram of folate monoglutamates. Conditions: column, Cosmosil 5Ph (5 μm, 150 × 4,6 mm I.D.); mobile phase, 50 m*M* potassium dihydrogen phosphate (pH 3.5) containing 15% methanol and 0.01 m*M* disodium EDTA; flow-rate, 0.5 ml/min; applied potential, +750 mV. Peaks (amount per 1.0 μl): A = H_4PteGlu (5 ng); B = 5-methyl-H_4PteGlu (3 ng); C = 10-formyl-H_4PteGlu (20 ng); D = 5-formyl-H_4PteGlu (20 ng); E = PteGlu (20 ng).

Separation of standard folate derivatives

Fig. 2 shows the elution profile of a mixture of H_4PteGlu, 5-methyl-H_4PteGlu, 5-formyl-H_4PteGlu, 10-formyl-H_4PteGlu and PteGlu. All these derivatives are baseline-separated when eluted under the conditions described in Experimental.

Linear range and detection limit

The response based on peak-height measurements was linear up to 20 pg/μl per injection, with excellent correlation coefficients. The linear regression equations and the coefficients of correlation were as follows: H_4PteGlu, $y = 2.09x + 0.30$ ($r = 0.998$); 5-methyl-H_4PteGlu, $y = 1.10x + 0.79$ ($r = 0.994$); 10-formyl-H_4PteGlu, $y = 0.280x + 0.006$ ($r = 0.999$); 5-formyl-H_4PteGlu, $y = 0.310x - 0.254$ ($r = 0.987$); PteGlu, $y = 0.202x + 0.138$ ($r = 0.994$), where y is a current response (nA) and x is the amount of sample injected (ng). The upper limit of the linear range was determined to be at least 0.5 μg per injection. This permits the convenient determination of folate concentration over the range found in biological samples. The detection limits at a signal-to-noise ratio of 3 are listed in Table I, along with the previously published results. The sensitivity of ED for PteGlu and its derivatives is much higher than that of

TABLE I

SENSITIVITY OF VARIOUS METHODS USING HPLC FOR DETECTION OF FOLATE MONOGLUTAMATES

Data represent the minimum levels detectable under the conditions described in each paper.

Detection method	Detection limit (pg per injection)					Reference
	H_4PteGlu	5-CH_3-H_4PteGlu	10-HCO-H_4PteGlu	5-HCO-H_4PteGlu	PteGlu	
UV (280 nm)	1500	1500	2500	2500	1000	12
Fluorometric (295/356 nm)	70	20	—	200	1000	14
L. casei assay*	30	50	25	30	25	13, 15
ED (+750 mV)	3.0	1.0	125	150	80	Present work

**Lactobacillus casei* assay, data in pg/ml.

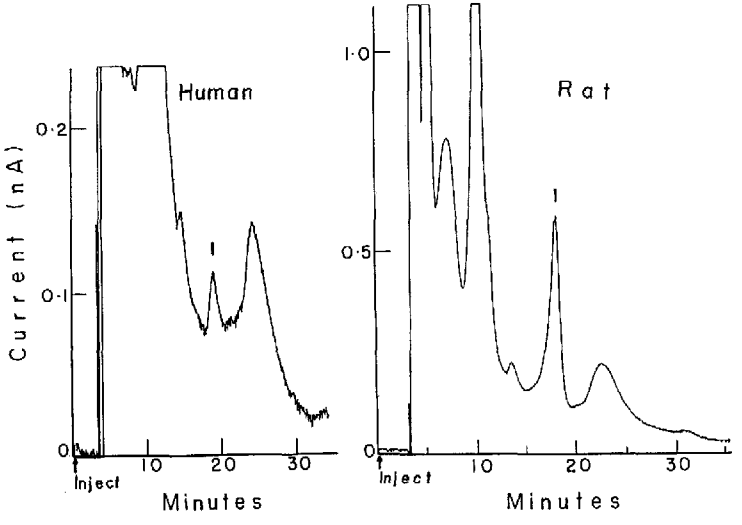

Fig. 3. Chromatograms of serum extracts from a human subject and a rat. Conditions as in Experimental. Peak I = 5-methyl-H_4PteGlu.

UV or fluorescence detection. HPLC—ED for H_4PteGlu and 5-methyl-H_4PteGlu is ten to fifty times more sensitive than the microbiological assay listed. This method allows most biological samples to be analysed with no preconcentration step.

Determination of folate concentration in serum

Human and rat sera were prepared and analysed by HPLC—ED as described under Experimental. Fig. 3 shows chromatograms obtained from typical human and rat sera. The peak corresponding to 5-methyl-H_4PteGlu is well resolved and was characterized on the basis of retention data and a constant voltammetric response between +350 and +550 mV (data not shown). The recovery of 5-methyl-H_4PteGlu added to the serum was 92.9% ± 4.9 (n=5) at a concentration of 10 ng/μl of sample. Serum levels of 5-methyl-H_4PteGlu before eating in human and rat were 2.8 ± 1.2 ng/ml (n=5) and 10.0 ± 3.1 ng/ml (n=5), respectively.

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