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Optimisation of chromatographic conditions for the determination of folates in foods and biological tissues for nutritional and clinical work

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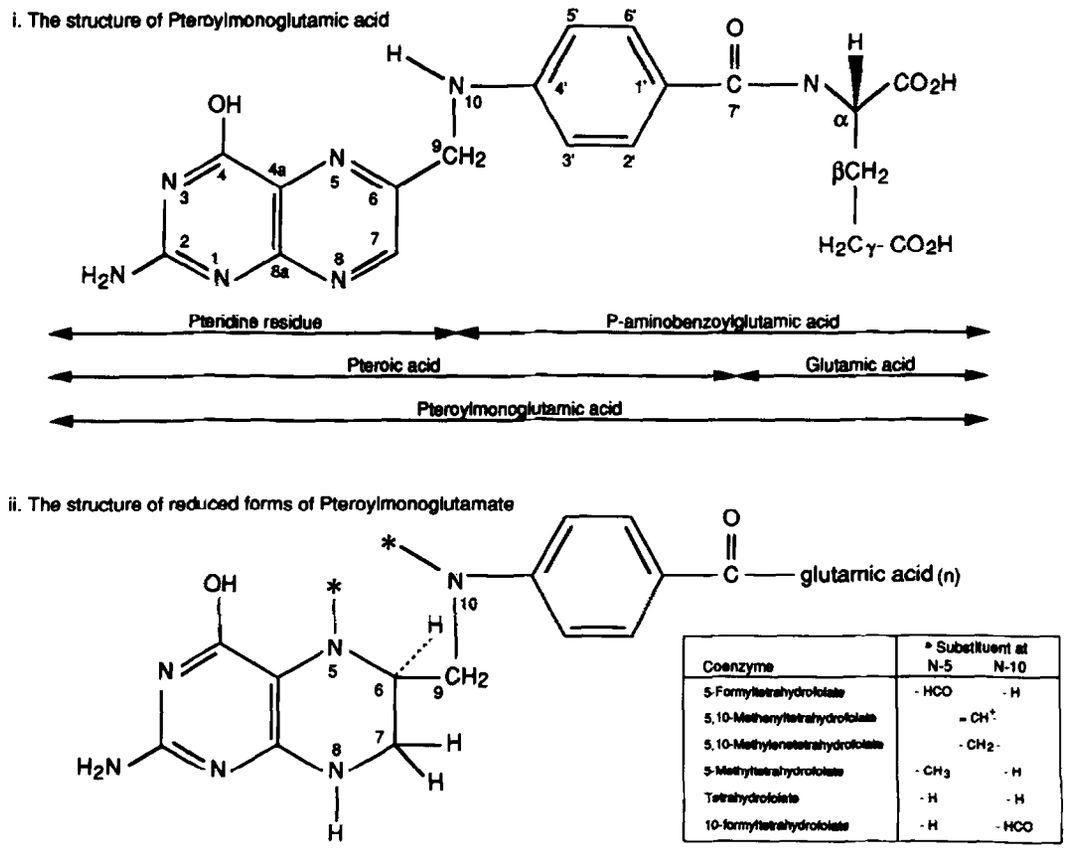
Recent studies implicate folate metabolism in the aetiology of heart disease, neural tube defects, malignant transformation and affective disorders. The paper reports a rapid, isocratic HPLC separation of 11 folylmonoglutamate compounds which should prove useful when adapted to the varied needs of analysts researching these and other specific areas. Also reported are the separation, UV spectra including λ_{max} values, fluorescence emission scans at an excitation wavelength of 295 nm and electrochemically derived hydrodynamic voltammograms with optimum oxidation voltages for *p*-aminobenzoylglutamate, tetrahydrofolate, 5-methyldihydrofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate, dihydrofolate, pteroylmonoglutamate and 5,10-methylenetetrahydrofolate. In particular, 5-methyltetrahydrofolate, the main food folate and form of the vitamin found in plasma, can be measured easily by electrochemical detection using a low and highly selective voltage of 450 mV. This reduced folate is also readily detected fluorimetrically using an excitation wavelength of 295 nm and measuring emission at 365 nm. Electrochemical and fluorimetric detection offer equal sensitivity for 5-methyltetrahydrofolate measurement (300 pg on column). No other folate studied could be measured down to this level using fluorimetric detection under the described conditions. At pH 3·5, folate coenzyme λ_{max} for UV detection varies between 267 and 300 nm with 5,10-methenyltetrahydrofolate giving maximum absorption at 355 nm. UV measurement of 5-methyltetrahydrofolate is approaching an order of magnitude less sensitive than the former methods of detection. However, for *in vitro* studies, particularly in the form of a photodiode array, UV detection is a particularly useful tool. For cerebrospinal fluid, plasma, erythrocyte or food measurement of $5CH_3$-H_4PteGlu, electrochemical or fluorimetric detection is recommended; whilst for pharmacokinetic studies of plasma 5CHO-H_4PteGlu during methotrexate rescue therapy, electrochemical or UV detection is most appropriate. For analysis of plasma PteGlu following supplementation, or in food stuffs, UV detection offers the best measurement technique. The information presented should help address the major problem of trace folate analysis by HPLC, that is the need to combine high sensitivity with optimum selectivity in studying complex matrices such as physiological fluids, tissue preparations and food samples.

INTRODUCTION

The various coenzymes of folic acid facilitate the transfer of one-carbon units from donor compounds to biosynthetic pathways. Folic acid itself (PteGlu) is a stable synthetic substance which is merely the parent molecule of a large family of chemically similar, highly labile trace compounds. These naturally occurring folates may differ in (i) the state of oxidation of the pteridine ring; (ii) the nature of the one-carbon substituents at the N5 and N10 positions; and (iii) the number of glutamic acid residues which are linked one to another via gamma glutamyl linkage to form an oligo-γ-glutamyl chain (see Scheme 1).

This multiplicity of form available to folyl derivatives, coupled to disadvantages associated with more classical analytical methods, has led to confusion over the biological occurrence and role of reduced folates. Indeed of all the vitamins, folic acid probably still represents the greatest challenge to contemporary bioanalysts involved with the clinical and food sciences.

In order to investigate folic acid homeostasis in a direct and specific manner, which is difficult with *Lactobacillus*



Scheme 1. Structure of folylmonoglutamates.

casei and radiometric binding assays alone, this laboratory has pioneered a sensitive and specific HPLC-electrochemical method for the determination of endogenous $5CH_3$-H_4PteGlu in plasma using solid phase extraction with internal standardisation (Lucock *et al.*, 1989*a*). This technique has been used successfully to investigate aspects of folate metabolism in cerebrospinal fluid (CSF), liver and plasma (including absorption kinetics) (Lucock *et al.*, 1989*a*,*b*,*c*, 1993*a*, 1994*a*) and has been adapted for measuring the physico-chemical parameters which influence $5CH_3$-H_4PteGlu degradation (Lucock *et al.*, 1993*b*, 1994*b*). Given the present resurgence of interest in one-carbon metabolism, the present paper reports the full potential of this useful chromatographic separation, giving physical data which should prove useful to analysts tackling this most difficult area of study.

Chromatography is described which can discriminate 11 folymonoglutamates and related compounds. Accurate UV spectra are given for these compounds in pure form using photodiode array detection. In addition, hydrodynamic voltammagrams and fluorescence scans of the same molecules provide useful information in developing sensitive and selective trace analyses of specific folylmonoglutamates using electrochemical and fluorescence detection, respectively. The relative sensitivities of UV, electrochemical and fluorescence detection for measuring the main extracellular folate — $5CH_3$-H_4PteGlu — and other congeners are given, as are other useful information.

MATERIALS AND METHODS

Chemicals

(6R,S)5-Methyltetrahydrofolic acid, calcium salt ($5CH_3$-H_4PteGlu); (6R,S)5,10-methenyl-5,6,7,8-tetrahydrofolic acid–HCl ($5,10CHH_4$PteGlu); (6R,S)5-methyl-5,6-dihydrofolic acid, calcium salt ($5CH_3$-H_2PteGlu); (6R,S) 5,10-methylene-5,6,7,8-tetrahydrofolic acid, magnesium salt ($5,10CH_2$-H_4PteGlu); (6R,S) 5,6,7,8-tetrahydrofolic acid-trihydrochloride (H_4PteGlu) and *p*-aminobenzoyl-L-glutamic acid (P-ABG) were purchased from Dr B. Schircks (Jona, Switzerland). Pteroylmonoglutamate (PteGlu), aminopterin, 7,8-dihydrofolic acid (H_2PteGlu), and folinic acid, calcium salt (5CHO-H_4PteGlu) were purchased from Sigma Chemical Co. Ltd (Poole, Dorset, UK). Teropterin ($PteGlu_3$) was a gift from Lederle. All other chemicals including those used to make the mobile phase are as previously described (Lucock *et al.*, 1989*a*).

Solutions

All aqueous solutions were prepared from filtered, deionised and double-distilled water obtained from an aquatron WD1-W4D water deioniser/still supplied by J. Bibby Science Products Ltd (Stone, Staffs, UK). Small volume stock solutions of all folates were prepared at a concentration of 1 mg/ml and snap frozen. Working solutions were prepared by thawing microlitre

volumes and diluting to a concentration within the range 10 ng/ml–100 μg/ml according to need. Care and alacrity with this approach can avoid the need for any antioxidant, although for quantitative measurement of H_2PteGlu or H_4PteGlu a 100-fold molar excess of ascorbate was used when preparing stock solutions. For the analysis of biological samples this separation can use DL-dithiothreitol (DTT), as described in Lucock *et al.* (1989*a*, 1993*b*), or ascorbate (Lucock *et al.*, 1989*a*). Antioxidants, however, rapidly convert $5CH_3$-H_2PteGlu to $5CH_3$-H_4PteGlu. At pH 6·4 and 25°C using ascorbate at a molar ratio of 1000 : 1 to reduce $5CH_3$-H_2PteGlu the time for 50% maximal conversion is 273·65 min, a process 36% efficient ($5CH_3$-H_4PteGlu formation rate constant K± confidence interval = −0·0025; −0·0088–+0·0039). At pH 3·5 and 25°C using the same ratio of ascorbic acid to reduce $5CH_3$-H_2PteGlu, the time for 50% maximal conversion to $5CH_3$-H_4PteGlu is 1·25 min, a process 100% efficient (K ± Cl = −0·5566; almost instantaneous two-point curve). Thus, antioxidants should not be used with $5CH_3$-H_2PteGlu. However, the following chromatographic system is ideally suited for use with either ascorbic acid or DTT when using certain folyl coenzymes, particularly H_2PteGlu, H_4PteGlu and $5,10CH_2$-H_4PteGlu which are extremely labile. These antioxidants should be used selectively since ascorbic acid is most effective under acid conditions (see above), whilst DTT protection is optimal under neutral conditions (Lucock *et al.*, 1989*a*, 1993*b*). A full description of $5CH_3$-H_4PteGlu stability in the presence and absence of antioxidants is given in Lucock *et al.* (1993*b*, 1994*b*).

Solutions prepared as described gave single chromatographic peaks, indicating probable purity, i.e. for $5CH_3$-H_4PteGlu, 0% $5CH_3$-H_2PteGlu was detected under chromatographic conditions which resolve both components. This is important since in the authors' experience $5CH_3$-H_2PteGlu represents the major impurity in this product from certain suppliers. PteGlu, however, did contain P-ABG as an impurity and H_2PteGlu can be particularly impure containing both P-ABG and PteGlu. Purity based on spectral analysis is discussed later.

Equipment and chromatographic conditions

The high-performance liquid chromatograph (HPLC) system is based on that previously described (Lucock *et al.*, 1989*a*), and modified according to Lucock *et al.* (1993*b*, 1994*b*).

The hardware consisted of a Model 6000A pump, U6K injector and (a) a 996 photodiode array detector, or (b) a model 470 scanning fluorescence detector (Waters Assoc., Watford, UK), or (c) an EG and G Model 400 electrochemical detector (Princeton Applied Research, Princeton, NJ, USA.)

Although the following chromatographic conditions are similar to those previously reported (Lucock *et al.*, 1989*a*, 1993*b*, 1994*b*), their potential for separating a large number of folylmonoglutamates has not been reported. The following separation is in fact capable of discriminating between at least 11 folylmonoglutamate compounds: A Waters Associates Nova-Pak phenyl stainless-steel column (7·5 cm × 3·9 mm. i.d.; 4 μM spherical bonded silica) was protected by a LiChroCART, LiChrospher-5μM C18 guard column (E. Merck, Darmstadt, Germany). Fresh mobile phase consisting of 15% (v/v) methanol in 0·05 M KH_2PO_4 (pH adjusted to 3·5 with *ortho*-phosphoric acid) was filtered through a 0·45 μM regenerated cellulose filter (Sartorius, Göttingen, Germany). The separation of folylmonoglutamates was performed at 25°C with a flow of 0·4 ml/min. Detection was by one of the following.

(a) Electrochemical, where a glassy carbon oxidative flow cell containing a 20 μM gasket (type TG-2M) was used. The electrode potential was held at 450 mV versus Ag/AgCl and the current measured down to 0·5 nA sensitivity.

(b) Fluorimetric detection; selective fluorimetric detection of folates was achieved with an excitation wavelength of 295 nm and emission setting of 365 nm. The optimum setting for giving an acceptable signal to noise ratio for sensitive detection was an attenuation of 4 with a gain value of 10.

(c) UV detection: this is not a realistic option for trace $5CH_3$-H_4PteGlu analysis. However photodiode array detection (PAD) is a useful tool for the characterisation, purity analysis, identification of, and spectral differentiation of folylmonoglutamates. PAD data was collected between 200 and 400 nm at 1·2 nm optical resolution in order to discriminate fine structural details of the similar folylmonoglutamate spectra. Evolving chromatograms were monitored at 280 nm and post-analysis routines were achieved using millennium 2010 chromatography manager software (Waters Assoc., Watford, UK).

Electrochemical scanning

Hydrodynamic voltammagrams were generated by using a half cycle scan which raised the applied voltage from 0 to 1250 mV at 20 mV/s. The lowest voltage at which a significant oxidation current is detected (usually at or just below the plateau of the first major signal response) is the best compromise between detection sensitivity (for analyte) and selectivity (from other oxidisable molecules).

Fluorescence scanning

Emission spectra between 305 and 700 nm were generated by an excitation wavelength of 295 nm.

RESULTS

Figure 1(a) and (b), show the chromatographic separation and photodiode array detection at 280 nm of 11

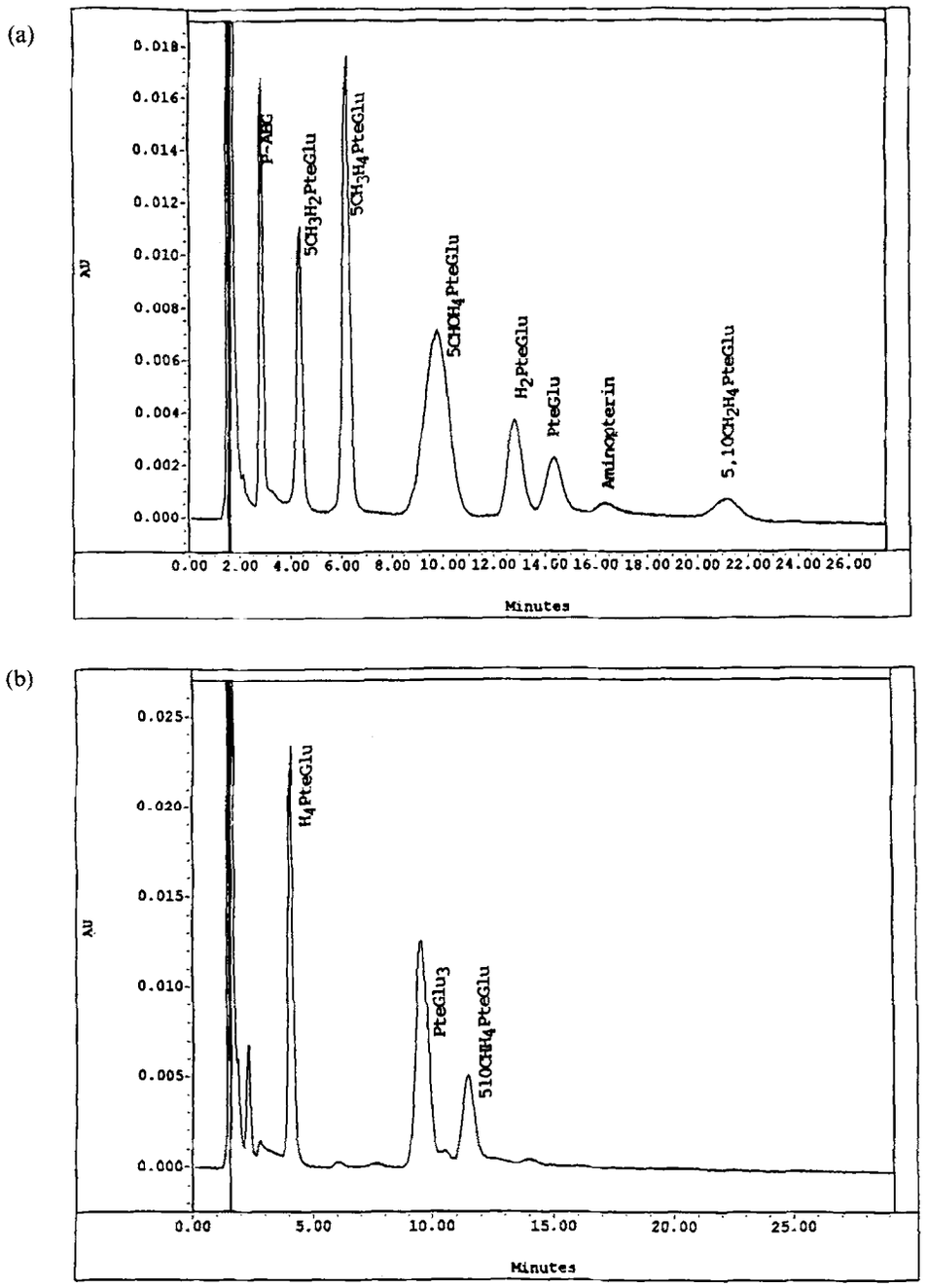


Fig. 1. Chromatographic separation of 11 folylmonoglutamates and related compounds at 280 nm (each compound represents 100 pmol on column). Trace 1 (b) shows folates which coelute with the folate compounds in trace 1 (a). The conditions are as described in Materials and Methods. Because of the ascorbate used to protect H_2PteGlu, H_4PteGlu and 5,10CH_2H_4PteGlu from oxidation, a moderate percentage (45%) of 5CH_3H_2PteGlu has been converted to 5CH_3H_4PteGlu.

equimolar folylmonoglutamates and related compounds (100 pmol on column for each molecule). The main chromatograph (Fig. 1. (a)) shows baseline resolution of P-ABG (2·82 min), 5CH_3-H_2PteGlu (4·32 min), 5CH_3-H_4PteGlu (6·16 min), 5CHO-H_4PteGlu (9·75 min), H_2PteGlu (12·79 min), PteGlu (14·31 min), aminopterin (16·25 min) and 5,10CH_2-H_4PteGlu (21·26 min). The chromatograph in Fig. 1(b) shows unresolved folates which have retention times of 4·063, 6·04 and 11·43 min for H_4PteGlu, $PteGlu_3$ and 5,10CHH$_4$PteGlu, respectively. The coelution of $PteGlu_3$ and 5CHO-H_4PteGlu is probably not important since $PteGlu_3$ is only likely to be measured in conjunction with PteGlu in assays for γ-glutamylcarboxy-peptidase (conjugase) activity, and these two molecules are well resolved. Fortunately H_4PteGlu and 5CH_3-H_2PteGlu can be discriminated by fluorimetric detection using an excitation wavelength of 295 nm and emission wavelength of 365 nm since only H_4PteGlu fluoresces (see Fig. 2 — fluorescence emission scans of folylmonoglutamates which clearly demonstrates that tetrahydro forms of the vitamin fluoresce better than dihydro forms, P-ABG being an exception whilst 5,10CHH$_4$PteGlu has poor absorption at this excitation wavelength). Furthermore, Fig. 3 (UV spectra of folylmonoglutamates between 200 and 400 nm) shows

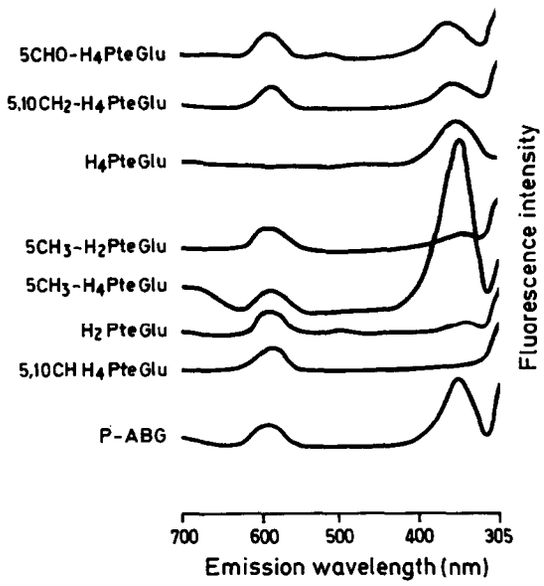


Fig. 2. Fluorescence emission scans resulting from excitation of folylmonoglutamates at 295 nm. Scan range 305–700 nm.

significant spectral resolution between H_2PteGlu and 5,10CHH_4PteGlu with λ_{max} being 280 and 355 nm, respectively, therefore again differentiation between partially coeluting peaks is possible. This figure also demonstrates that at this pH, three basic spectral forms exist: (i) H_4PteGlu and 5CH_3-H_4PteGlu each provide a wide wavelength range over which absorption is maximal (i.e. 260–300 nm); (ii) 5,10CHH_4PteGlu, H_2PteGlu and PteGlu (and $PteGlu_3$) offer significant absorption between 320 and 355 nm, although the latter three have a λ_{max} at 280 nm; (iii) all other folates have a narrow wavelength maxima ranging from 270 to 300 nm depending on the particular coenzyme. PAD software gives a purity threshold value for each peak, which is a measure of optical noise and solvent induced spectral changes. When purity angle, which indicates the relative spectral homogeneity across a peak, is less than the purity threshold, a pure peak is indicated. Table 1, which gives a useful summary of chromatographic and detection phenomena associated with folylmonoglutamates gives the purity angle/threshold value ratio, an indicator of compound purity. This measure of purity is important since all the spectra in Fig. 3 are derived from photodiode array detection of separated chromatographic peaks resolved under the conditions in Fig. 1, i.e. they are homogenous in character and therefore demonstrate the chromatographic selectivity for this separation of authentic materials. The ability to check for purity in this way is advantageous since in real samples coeluting peaks may affect quantitation. Clearly, in the mobile phase environment described, subtle differences in one-carbon substituent and oxidation state produce differentiable folylmonoglutamate spectra, which in the context of PAD and in combination with retention time, provides a unique 'fingerprint' for comparison. Other workers have used spectral features to discriminate between folates using PAD (Selhub, 1989). In one such study, clusters of variously substituted reduced folates coelute, and it is the spectral features of, in this case, folylpolyglutamates that

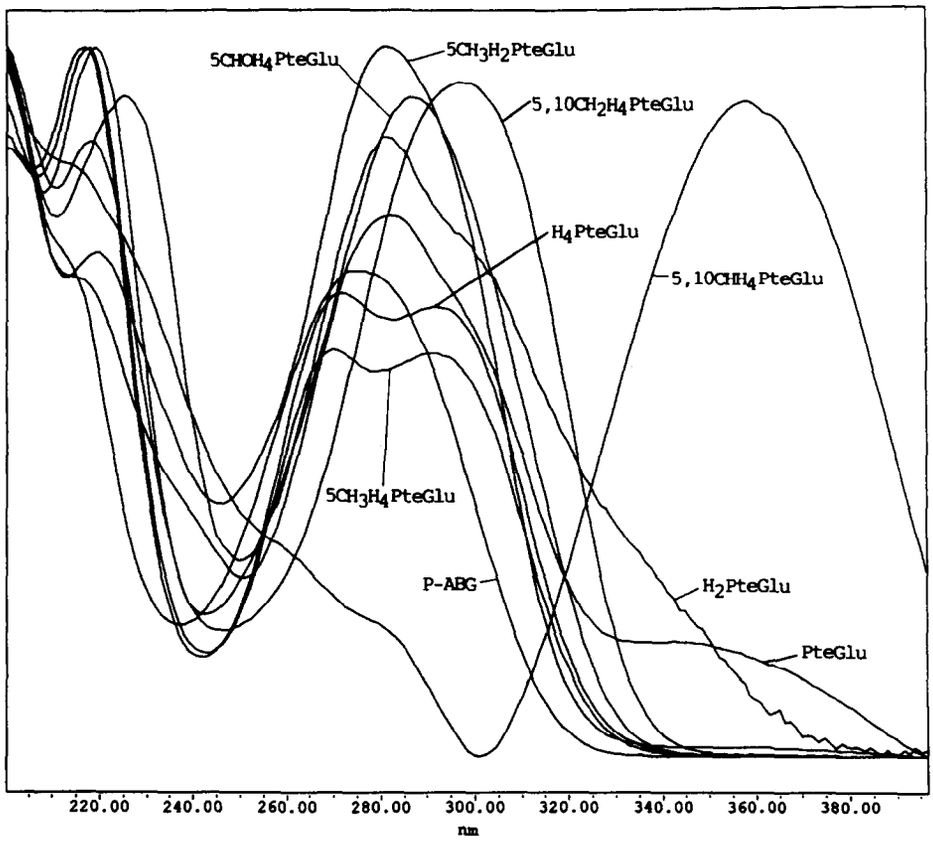


Fig. 3. UV spectra (200–400 nm) of folylmonoglutamates following chromatographic separation in the described pH 3·5 mobile phase. Optical resolution = 1·2 nm.

Table 1. Physico-chemical data for folylmonoglutamates useful in developing methods for the chromatographic speciation of these compounds

Folymono-glutamate	Chromatographic retention time (min) at 0·4 ml/min	λ_{max} (nm) for UV detection between 240 and 400 nm (Relative absorptivity (%) is given in parentheses)	Optimum voltage for electrochemical detection using a glassy carbon electrode (mV)	Optimum emission wavelength (nm) at an excitation wave length of 295 nm[a]	Purity angle: Purity threshold value <1·0 implies peak homogeneity
P-ABG	2·82	272 (100)	900	(1) 358 (2) 609	0·23
$5CH_3$-H_2PteGlu	4.32	276 (100)	No signal below 1000	607	0·10
H_4PteGlu	4·06	267 (100) 290 (97)	400	358	0·58
$5CH_3$-H_4PteGlu	6·16	267 (100) 290 (99)	400	(1) 358 (2) 607	0·18
5CHO-H_4PteGlu	9·75	285 (100)	600	(1) 372 (2) 607	0·86
5,10CHH_4PteGlu	11·43	(1) 355 (100) (2) 255 (36) (3) 280 (26)	900	607[a]	0·08
H_2PteGlu	12·79	(1) 280 (100) (2) 300 (82) (3) 325–350 (27–46)	400	607	0·04
PteGlu	14·31	(1) 280 (100)[b] (2) 300 (82) (3) 350 (25)	850	No emission in the 350–365 region	0·06
5,10CH_2H_4PteGlu	21·26	300 (100)	500	(1) 363 (2) 607	0·01

[a]Excitation λ is not optimum.
[b]Spectral characteristics of $PteGlu_3$ are almost identical to PteGlu.

Table 2. Comparison of UV fluorescence and electrochemical detector sensitivity towards folylmonoglutamates and related compounds

Congener	Amount on column, chromatographic conditions as described				Common reasons for measurement
	Minimum UV detection limit equivalent to 0·0003 au	Minimum electro-chemical detection limit		Minimum fluorescence detection limit at 295 nm excitation/ 365 nm emission	
		450 mV	800 mV		
p-ABG	530 pg	[a]	[a]	3·12 ng	Stability studies on C9-N10 cleavage.
$5CH_3H_2$PteGlu	1·2 ng	[a]	[a]	[b]	Stability studies, $5CH_3$-H_4PteGlu purity analysis.
H_4PteGlu	790 pg	1·1 ng	440 pg	4·9 ng	Biochemical studies.
$5CH_3$-H_4PteGlu	1·7 ng	300 pg	240 pg	300 pg	Food, plasma, whole blood and pharmaceutical analysis. Can be used for methotrexate rescue.
$PteGlu_3$	2·3 ng	—[c]	—	—	Synthetic substrate useful in biochemical studies.
5CHOH_4PteGlu	3·8 ng	No signal	800 pg	18·8 ng	Plasma and pharmaceutical analysis in relation to methotrexate rescue. Is a precursor for 10CHOH_4PteGlu.
5,10CHH_4PteGlu	7·4 ng (280 nm) 2·4 ng (355 nm)	[a]	[a]	[b]	Precursor for synthesis of 10CHOH_4PteGlu.
H_2PteGlu	2·7 ng	1·7 ng	180 pg	[b]	Biochemical studies.
PteGlu	3·3 ng	[a]	[a]	[b]	Found in food and plasma following supplementation. Pharmaceutical analysis.
Aminopterin	26·4 ng	—	—	—	Antifolate.
5,10CH_2H_4PteGlu	9·6 ng	2 ng	—	52·4 ng	Biochemical studies.

[a]Oxidation state or occurrence makes this form of detection inappropriate.
[b]Inappropriate form of detection or excitation wavelength.
[c]—, Not studied.

are discriminatory; 280 nm measures general folate activity, 350 nm measures dihydro and stable pteroyl derivatives and 258 nm measures 10 formyl forms (not described here because of their unavailability in pure form and need for high concentrations of antioxidant for synthesis from 5,10CHH$_4$PteGlu). Despite its many virtues, for the most important biological folate, 5CH$_3$-H$_4$PteGlu, UV detection is the least sensitive form of measurement and for trace analysis, electrochemical or fluorescence detectors are often employed. This is demonstrated in Table 2, which compares these three forms of HPLC detection in terms of sensitivity towards a range of folylmonoglutamates — clearly not all folates exhibit the same detector response as 5CH$_3$-H$_4$PteGlu, and in many cases UV may be the best form of detection.

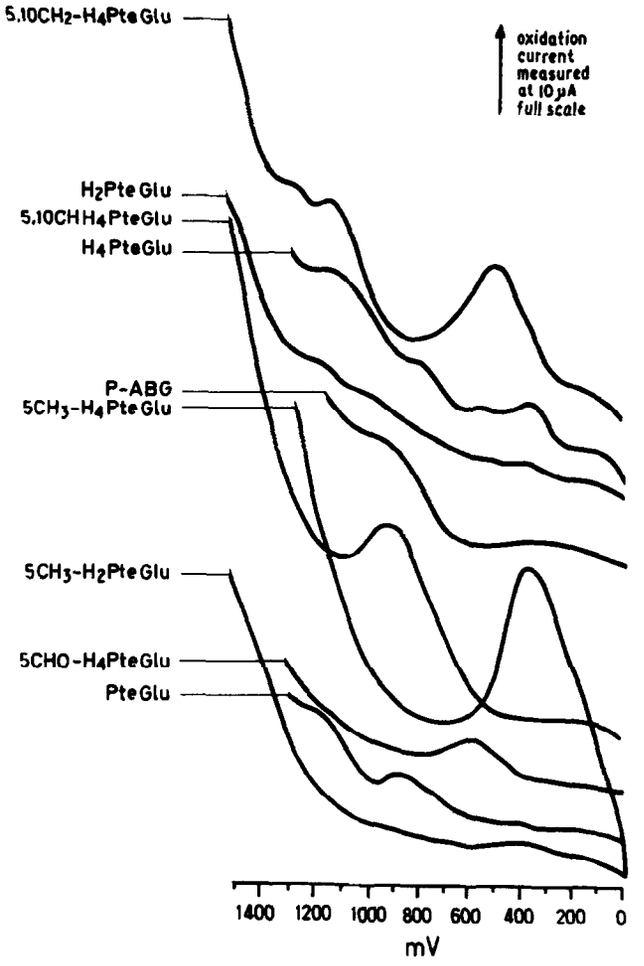


Fig. 4. Electrochemically derived voltammagrams for folylmonoglutamates. Half cycle scan rate 20 mV/s at 10 μA full-scale.

ECD has been used extensively for folate analysis (Lucock *et al.*, 1989*a*,*b*,*c*, 1993*a*,*b*, 1994*a*,*b*) and has been an extremely useful tool for trace 5CH$_3$-H$_4$PteGlu analysis. Figure 4 shows a series of scans describing the hydrodynamic voltammagrams for nine folylmonoglutamates. Figure 5 shows how electrochemically derived voltammagrams, like spectra obtained using PAD, can identify a compound. In the example given, similar voltammagrams of authentic 5CH$_3$-H$_4$PteGlu and endogenous 5CH$_3$-H$_4$PteGlu extracted from plasma identify extremely low concentrations of 5CH$_3$-H$_4$PteGlu in a real biological sample using criteria other than simply retention time. The difference between 5CH$_3$-H$_4$PteGlu voltammagrams in Figs 4 and 5 are due, respectively, to the oxidation of a single sample being subjected to a continuous elevation in voltage of 20mV/s — without chromatographic separation vs several injections of the same sample being injected into and separated on a liquid chromatograph. Following chromatographic separation each injection of analyte is detected at a separate voltage. Clearly this approach for the verification of 5CH$_3$-H$_4$PteGlu peak identity at trace levels has much to commend it and offers electrochemical identification at levels too low for PAD to work at.

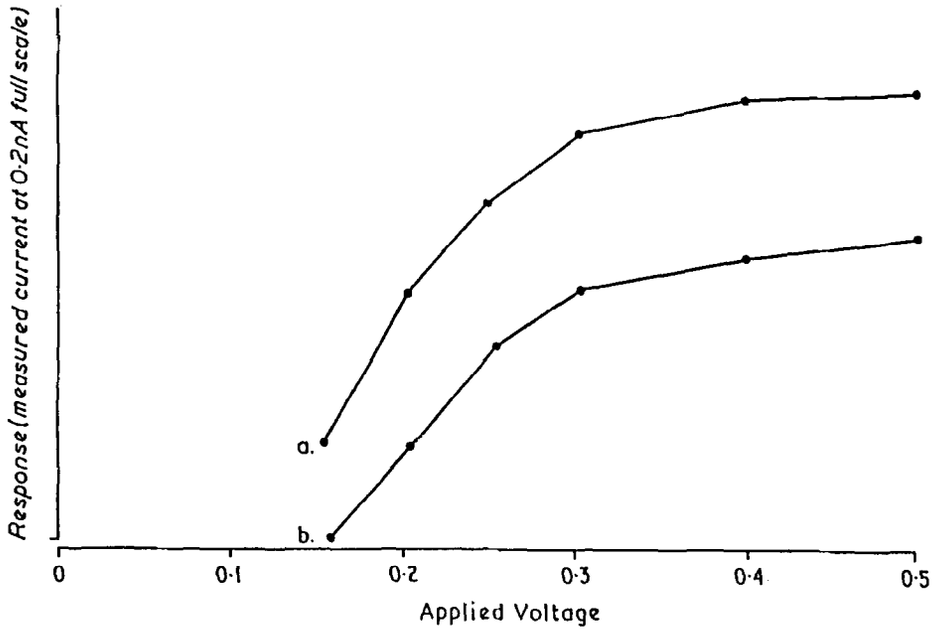


Fig. 5. Hydrodynamic voltammagrams for (a) authentic 5CH$_3$-H$_4$PteGlu, and (b) the electrochemically active substance in plasma with peak corresponding to the retention time of 5CH$_3$-H$_4$PteGlu (conditions as detailed in Lucock, 1989*a*).

DISCUSSION

What is clear from these data is that in general oxidised folylmonoglutamates do not lend themselves to either native fluorescence or low voltage, and hence selective electrochemical detection, whilst reduced tetrahydro forms do. Exceptions to this are reduced 5,10CHH_4PteGlu which does not fluoresce at an excitation wavelength of 295 nm, not surprising given a λ_{max} of 355 nm (see Fig. 3), and P-ABG which does. Furthermore, H_2PteGlu can be detected electrochemically. The fluorescence scan for PteGlu has not been included in Fig. 2 since our sample produced spurious results. It did not, however, show an emission at 365 nm (excitation wavelength 295 nm) which agrees with the findings of others who employ an oxidative post-column derivatization system for measuring PteGlu using fluorimetric detection. Briefly in this method hypochlorite cleaves PteGlu (and H_2PteGlu and H_4PteGlu) to fluorescent pterins. When this reaction is performed downstream from a fluorometer measuring the native fluorescence of reduced folates, all folates can be measured fluorimetrically (Gregory *et al.*, 1984). Based on the findings in Table 2, and considering sensitivity only, UV would appear to be the detection mode of choice for P-ABG, 5CH_3-H_2PteGlu, PteGlu and 5,10CHH_4PteGlu, although P-ABG can also be measured at low levels fluorimetrically. ECD is the detection mode best suited to H_4PteGlu, H_2PteGlu, 5CH_3-H_4PteGlu, 5,10CH_2-H_4PteGlu and 5CHO-H_4PteGlu, although only the former four folates offer the selective detection afforded by a low oxidation voltage (note: H_4PteGlu and H_2PteGlu sensitivity improve at higher, less selective voltages and both molecules can be measured at low levels using UV detection). Fluorimetric detection is of equal sensitivity to ECD with respect to 5CH_3-H_4PteGlu, but less sensitive for H_4PteGlu, 5CHO-H_4PteGlu and 5,10CH_2-H_4PteGlu. With the exception of 5CH_3-H_4PteGlu, fluorescence proved to be a less sensitive form of detection than UV. However, like P-ABG, H_4PteGlu can be measured at quite low levels using fluorescence, although even for this folate UV still offers better sensitivity. Clearly, the relative sensitivity of different forms of detection to the various folates will be dependent upon factors which may vary between the many HPLC techniques reported. These include mobile phase conditions; supporting electrolyte, pH, organic modifier, instrumentation design especially optical parameters such as lamp energy, although specific excitation/emission wavelengths are also important as is cell design in electrochemical detection.

The authors' experience with ECD to measure 5CH_3-H_4PteGlu in a variety of biological samples is reported above. By comparison, fluorimetric detection has equal potential for analysing complex samples since it provides substantial specificity (certainly greater than UV and possibly better than ECD) coupled with good sensitivity. Although utilising the present mobile phase conditions, and in terms of sensitivity, only 5CH_3-H_4PteGlu benefits from this form of detection and then is only the equal of ECD; UV < fluorescence = ECD. 5CH_3-H_4PteGlu being readily quantifiable down to 1·7 ng, 300 pg and 300 pg on column, respectively. However, based on a signal to noise ratio of 2·5, ECD and fluorescence methods can actually visualise a peak of 100 pg 5CH_3-H_4PteGlu on column. Apart from detection of 5CH_3-H_4PteGlu, fortuitously the most important folate coenzyme, fluorimetric sensitivity to folates using the described conditions is generally inferior to UV sensitivity, but should benefit from greater overall selectivity. Others have found fluorimetric detection to be particularly useful in trace analysis of plasma 5CH_3-H_4PteGlu and even erythrocyte folate (Leeming *et al.*, 1990).

Taking into account the findings here and those of others, it is therefore possible to conclude that for the measurement of endogenous 5CH_3-H_4PteGlu in real samples such as CSF, plasma, erythrocytes or food, ECD or fluorimetric detection would be most suitable. For analysing pharmacological 5CHO-H_4PteGlu in plasma following methotrexate rescue therapy, the selection of ECD or UV detection would be most appropriate, whilst UV detection should be able to detect PteGlu in supplemented food or in plasma following doses high enough to saturate its conversion to 5CH_3-H_4PteGlu during absorption (Lucock *et al.*, 1989*b*).

Ultimately the form of detection adopted has to depend upon

(a) the amount of analyte present (sensitivity);
(b) the matrix it is in (selectivity); and
(c) the specific folate(s) of interest (tailor detection for optimum oxidation potential/native fluorescence criteria/λ_{max}).

Although direct folate analysis can be fraught with difficulty, a pragmatic approach does exist. Given the ability to hydrolyse intracellular and food folylpolyglutamates with various conjugase preparations (Finglas *et al.*, 1993), the described separation has potential not only for direct analysis but also for a fourth form of detection — offline bioassay using either *Lactobacillus casei* microbiological assay, radio assay or the more recent protein binding assay (Finglas *et al.*, 1988). This approach has been used with success for both CSF (Lucock *et al.*, 1993*a*) and plasma analysis (Lucock *et al.*, 1989*b*).

Improving ways to chromatographically speciate folylmono and polyglutamates will open up a new frontier in understanding one-carbon metabolism in health and disease. Existing bioassays, whether *L. casei* or radiometric binding assays, have disadvantages. Primarily they fail to differentiate the many possible forms of folate since on their own they are non-specific. Their response depends on the folate form used as calibration standard, the form present in the matrix under investigation (seldom is this the same as the calibration standard), pH of the medium, degree of folate degradation or interconversion (*L. casei* growth response probably differs between native and degraded forms of the vitamin (Lucock *et al.*, 1993*b*). The purity of standard

materials for bioassay is important; $5CH_3$-H_4PteGlu may contain significant $5CH_3$-H_2PteGlu, H_2PteGlu sometimes contains PteGlu and PteGlu may have P-ABG present (folates used from Dr B. Schircks appear to be of a particularly high standard). However, even if samples are pure on purchase, storage can lead to degradation. These impurities and degradation products could affect (a) pH dependant protein binding energies in radioassay, (b) test organism growth response in microbiological assay, and (c) quantitative calibration.

This paper shows that HPLC offers a way of ascertaining folate purity and demonstrates its potential for specific and sensitive folate analysis applicable to many important areas of clinical and nutritional study. For instance, the oxidation state and one carbon substitution profile of intra-erythrocyte/cellular folylpolyglutamates may provide answers to the neural tube defect (NTD) problem (Smithells *et al.*, 1983; Smithells *et al.*, 1989; MRC Vitamin Study Group, 1991; Czeizel & Dudas, 1992) and explain why periconceptional PteGlu supplementation has proven so effective in prevention of NTD (Lucock *et al.*, 1994*a*). It may explain why pharmacological $5CH_3$-H_4PteGlu is efficacious in treating affective disorders (Godfrey *et al.*, 1990; Crellin *et al.*, 1993) and facilitate investigation into how dietary folate deficiency influences malignant transformations such as cervical neoplasia (Schneider & Shah, 1989; Butterworth, 1993), bronchial metaplasia (Kamei *et al.*, 1993) and dysplasia and cancer in chronic ulcerative colitis (Lashner *et al.*, 1989); and perhaps with greatest potential benefit of all, will permit study into how $5CH_3$-H_4PteGlu dependent methylation lowers vasculotoxic homocysteine levels and reduces the rate of coronary heart disease (Kang *et al.*, 1987; Brattstrom *et al.*, 1988; Malinow *et al.*, 1989; Brattstrom *et al.*, 1990; Genest *et al.*, 1991; Lewis *et al.*, 1992; Mason & Miller, 1992; Stampfer *et al.*, 1992).

Given the present resurgent interest in folate metabolism, the basic chromatographic data presented here may be useful when adapted to the specific needs of individual researchers and analysts.

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