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Optimized high-performance liquid chromatographic procedure for the separation and determination of the main folacins and some derivatives

II. Extraction method and application to rat liver

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ABSTRACT

Different methods for the extraction of folacins from biological materials and the hydrolysis of pteroylpolyglutamates prior to high-performance liquid chromatography were investigated. Acetone precipitation of proteins led to higher extraction rates of folates from biological materials as examined by using an endogenous labelling technique. It also caused less destruction of some folates but could not be combined with subsequent hydrolysis of pteroylpolyglutamates. Pteroylpolyglutamates were hydrolysed by a partially purified suspension of pteroylpolyglutamates hydrolase (PPH). Comparative studies on the folate content of rat liver revealed that complete hydrolysis of polyglutamates could also be achieved by incubating the homogenized tissue at 37°C to allow endogenous PPH to act. This procedure causes interconversions of folates and is therefore not suitable for the analysis of biological materials.

INTRODUCTION

The determination of folacins is still an analytical problem because of the high lability of the substances and their low content in biological materials. The chromatographic system we presented in a previous paper [1] allows the fast and sensitive separation and determination of the main folacin monoglutamates and the synthetic derivatives dichlorofolic acid and methotrexate which were used as internal standards.

In this paper we review critically the methods for the extraction of folacins from biological materials and the hydrolysis of the pteroylpolyglutamates because many problems in folacin analysis arise directly from disturbances caused by the extraction method and by the hydrolysis of the pteroylpolyglutamates [2]. On the one hand the folates should be liberated quantitatively from protein binding during the extraction procedure, and on the other hand the rate of conversion to degradation products or to other metabolites should be minimal. For this reason all media must contain stabilizing agents, *i.e.*, antioxidants such as ascorbic acid [3–8], mercaptoethanol [9–

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13] or a combination of both [14]. Nevertheless as some folates show lability towards oxygen, a certain loss of substances can always be observed [13,15].

The most common technique used for the extraction of folates from biological materials is heat treatment at 80–100°C for 2–60 min [5–8,13,14]. The precipitation of proteins by trichloroacetic acid [16,17] or acetone [9] and freezing the tissue for cell cracking [13] has been used in only a few instances.

The pteroylpolyglutamates which represent the active forms of folacins must be converted into the corresponding monoglutamates before their chromatographic analysis [1]. The glutamyl residues are linked by the unique γ-peptide bond which is not attacked by conventional exo- and endopeptidases. The hydrolysis can only be achieved by pteroylpolyglutamate hydrolases (PPH, 'conjugases', E.C. 3.4.12.10), enzymes that are found in many tissues but which are not commercially available [18,19]. For this reason, folates are usually hydrolysed by incubating the homogenized tissue before heating [6,8] or by adding a more or less purified suspension of PPH [7,11,20].

The aim of this study was to test the applicability of heat treatment and acetone precipitation for the extraction of folates and their suitability for a subsequent hydrolysis of pteroylpolyglutamates. The application of the overall method to biological samples is reported.

EXPERIMENTAL

For the extraction buffer in all experiments we used a minor modification of the buffer proposed by Gregory *et al.* [7].It contained 0.1 *M* sodium acetate (pH 4.9) and 1% of sodium ascorbate as an antioxidant and was prepared freshly on the day of use.

Determination of the extraction efficiency

'Everted sacs' from rat small intestine [21] were incubated for 1 h in 25 ml of Krebs's hydrogencarbonate solution containing 5 m*M* glucose and 1μM potassium 3′,5′,7,9-[^{3}H]pteroylglutamate ([^{3}H]PteGlu, specific activity 1660 GBq/mmol) (Amersham, Braunschweig, Germany). The radiochemical purity was examined by high-performance liquid chromatography (HPLC) [1] and found to be 97%. When incubations were terminated the segments (ca 10 cm long) were opened, blotted on paper, homogenized in a glass–Teflon homogenizer with acetate buffer and adjusted to a final volume of 5 ml.

For acetone extraction, 1 ml of the homogenate was placed in a reagent tube and mixed with 2 ml of ice-cold acetone for 1 min. After flushing with nitrogen for 30 s, the tube was closed tightly and centrifuged for 30 min at 10 000 *g*. The resulting supernatant was transferred to a second tube and the organic solvent was removed under a stream of nitrogen while the tube was kept in the cold. The radioactivity in the supernatant was measured by liquid scintillation counting and compared with that in the untreated homogenate.

Heat-treatment experiments were carried out in parallel with 1 ml of the same homogenates. The sample was heated in a water-bath at 95°C for 60 min after having been flushed with nitrogen. Subsequently it was immediately cooled on ice and centrifuged under the conditions mentioned above. The extraction efficiency was calculated in the same manner as in the acetone precipitation experiments.

In an additional series of experiments, heat treatment was performed prior to homogenization. In this instance the tissue was coarsely cut with scissors, covered with the buffer solution and immediately homogenized after heating.

Stability studies

Determinations of the substrate stability during the extraction procedure were carried out with standard solutions of different folates. Pteroylglutamic acid (Pte-Glu), dihydrofolic acid (DHF), tetrahydrofolic acid (THF), sodium 5-methyltetrahydrofolate (5-CH_3-THF), calcium 5-formyltetrahydrofolate (5-CHO-THF), methotrexate (MTX) and 3′,5′-dichlorofolic acid (DCF) were obtained from Sigma (Deisenhofen, Germany) and were dissolved in the extraction buffer to final concentrations of 50 pmol/ml for 5-CH_3-THF and 250 pmol/ml for all others. Volumes of 5 ml of these solutions were heated or mixed with acetone as described above. The contents of the different folates in the resulting supernatant were measured by HPLC with fluorimetric detection [1] and were compared with the folate content of untreated samples that had been assayed immediately.

Hydrolysis of pteroylpolyglutamates

A PPH suspension was prepared according to methods described previously [7,22]. The suspension (16 mg/ml of protein) was assayed by HPLC and found to be free from any folate compounds. In incubation experiments 0.4 ml of enzyme suspension was added to 1 ml of acetate buffer containing 2 pmol of different folates and incubated for 30 –120 min.

The hydrolytic capability of the enzyme suspension was tested by incubating extracted homogenates from cabbage and rat liver. A volume of 1 ml of the extraction supernatant was mixed with 0.4 ml of hog PPH and incubated for 30–90 min at 37°C. The increase in monoglutamates was measured by HPLC and compared with the monoglutamate content of samples that were assayed immediately after homogenization.

Application of the method to biological tissues

To show the validity of the overall method we determined the folate content of rat liver. The analysis was monitored using internal standards, DCF and MTX, both of which were added to each sample prior to extraction. Fig. 1 summarizes the extraction scheme used. For comparison we also analysed rat liver by a modified procedure: the liver was homogenized in the extraction buffer and then incubated for 60 min at 37°C to make it possible for the endogenous PPH to hydrolyse the pteroylpolyglutamates. The homogenate was then heated at 95°C for 60 min to liberate the folates, centrifuged (10 000 *g*, 30 min, 4°C) and the supernatant was analysed by HPLC.

In all instances determinations were performed using an external calibration graph. The results were corrected for the incomplete extraction and for the loss of substances as a consequence of heat destruction.

RESULTS AND DISCUSSION

Extraction procedure

Table I gives the results of the experiments concerning the extraction efficiency,

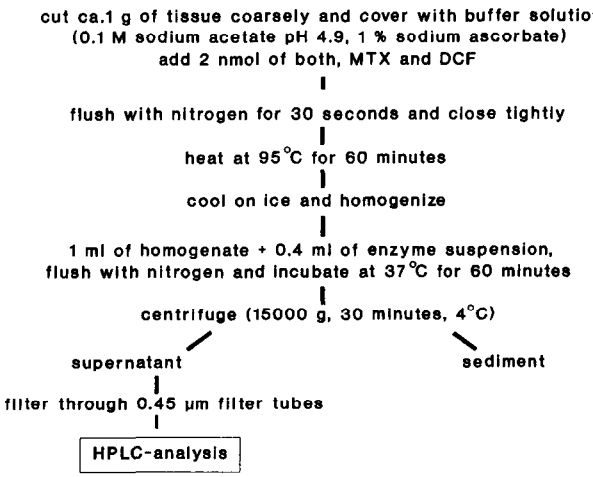

Fig. 1. Extraction of folates from biological samples and subsequent hydrolysis of polyglutamates. If it is intended to determine the content of "free" folate, *i.e.*, the folacin monoglutamates, 1 ml of the homogenate must be treated in the same way as described except for the hydrolysis with pteroylpolyglutamathydrolase.

indicating that acetone extraction was significantly better than heat treatment of the sample for extraction of folates from tissue. As can be seen, there was no significant difference between heating before and after homogenization.

The extraction efficiency is one important factor responsible for the precision of a method, but it is difficult to determine [23]. We chose an endogenous labelling technique to evaluate the extraction efficiency using intestinal tissue as a model matrix because our method was designed primarily to determine the folate content of the gut wall [24]. Chromatographic studies revealed that more than 75% of the original substrate [^{3}H]PteGlu had been converted into other folacin derivatives (Fig. 2), in-

TABLE I

EXTRACTION EFFICIENCY[a] OBTAINED BY HEAT TREATMENT OF THE SAMPLE COMPARED WITH AN ACETONE PRECIPITATION TECHNIQUE

Experiments were carried out as described under Experimental.

Method [b]	Extraction efficiency (%) (± S.D., $n = 11$)
Acetone precipitation	95.8 ± 10.7
Heat treatment[c]	78.7 ± 6.1
Heat treatment[d]	77.3 ± 7.2

[a] Extraction efficiency is the amount of ^{3}H-labelled folates found in the supernatant after centrifugation compared with the radioactivity of the whole sample.

[b] $P \leqslant 0.001$ between acetone precipitation and the two heat-treatment techniques.

[c] Heating was performed after homogenization of the tissue.

[d] Tissue was homogenized after heat treatment (final method).

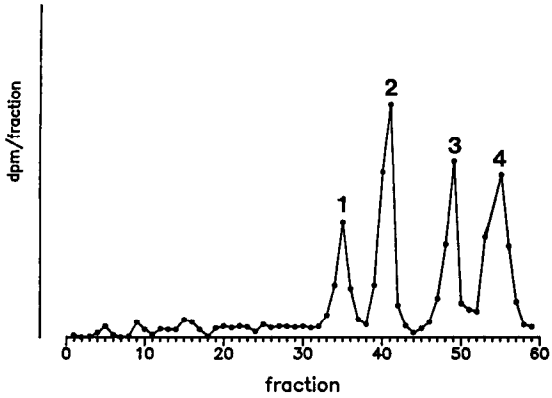

Fig. 2. Separation of radiolabelled folates from "everted sacs" of rat small intestine. Intestinal tissue had been incubated for 60 min with 1 μM [^{3}H]PteGlu and extracted as shown in Fig. 1. Separation conditions: column, Shandon Hypersil ODS (3 μm, 250 × 4.6 mm I.D.); mobile phase, gradient of (A) 5 mM potassium phosphate (pH 2.3) and (B) acetonitrile, from 7 to 13% B in 15 min and from 13 to 19% B in 3 min; flow-rate, 0.9 ml/min; temperature ambient. The solvent stream was collected in fractions of 0.3 ml and assayed by liquid scintillation counting. Peaks were identified by comparison with chromatograms that had been recorded using UV detection at 295 nm. Peaks: 1 = THF; 2 = 5-CH_3-THF; 3 = 5-CHO-THF; 4 = PteGlu.

dicating that the substrate was at least partially equilibrated with the endogenous folates.

Up to now only one attempt to determine the extraction efficiency for folates from rat liver has been described [11]. Using 1.5 M 2-mercaptoethanol as an extraction medium, about 90% of the activity was found in the supernatant after heat treatment of the sample.

We favour the endogenous labelling technique over the generally given 'recovery rates', which are easier to determine but which involve several problems. For example, they do not reflect the efficiency by which protein bonds are cleared because substrates added to tissue homogenate just before the extraction cannot be expected to behave like the endogenous substances. Further, as pointed out by others [7], the amounts of substrate usually added are often much higher than the natural substance content of the sample. Therefore, the recovery might increase because the relative amount of substrate included in the precipitate falls when higher substrate concentrations are used.

Our results confirm this assumption. After adding a very small amount (3.3 pmol) of [^{3}H]PteGlu to a homogenate of intestinal tissue, 70.9 ± 3.55% (n = 16) of the activity was found after heat treatment. In contrast, when 300 pmol of substrate had to be extracted 78.7 ± 6.1% (n = 11, $p \leqslant 0.001$) was found in the supernatant. However, additional results revealed that there were no significant differences when various folate contents between 300 and 2000 pmol/ml had to be extracted. Within this range the extraction efficiency was nearly constant and only showed non-directed variations between 76.2% and 80.1%. For this reason, the extraction efficiency can be assumed to be constant under these conditions. It should be mentioned that liver samples treated as shown in Fig. 1 contained about 2000 pmol/ml of folates in homogenate.

The efficiency of the extraction process, as judged by the amount of radioactivity in the supernatant, does not give any information about the loss of substances as a consequence of thermal or chemical degradation processes. We examined the stability of the different derivatives during the extraction procedure (Table II), which is the second factor determining the suitability of an extraction process.

The results indicate that PteGlu, DHF and 5-CHO-THF were destroyed less during acetone treatment. During heat treatment and also acetone precipitation no measurable increase in different folates could be observed, indicating that the loss of certain derivatives was due to complete degradation of these substances. The most labile derivative was found to be DHF, which was significantly better preserved using acetone treatment but which was nevertheless considerably destroyed. Additional studies showed that the extent of thermal destruction of DHF was about 90% after 10 min of heating, and the derivative could no longer be detected after 20 min. The fact that this compound was labile even under the relatively mild conditions during acetone treatment explains why it could hardly be found in natural materials [25]. Only Clifford and Clifford [10] have reported that DHF was the main folate in some foods, although they used heat treatment of the samples to extract tissue folates. We assume that these results are incorrect owing to the use of an inappropriate chromatographic system, *i.e.*, with a peak half-width of about 5 min and UV detection at 280 nm. Our data are consistent with those of O'Broin *et al.* [26], who reported that DHF was too labile to carry out systematic studies on its stability.

Hydrolysis of the pteroylpolyglutamates

Chromatographic analysis of pteroylpolyglutamates requires their hydrolysis to the corresponding monoglutamates [1]. This can be achieved by two general techniques. The fact that many tissues contain PPH allows the sample to be incubated after homogenization with its endogenous PPH [6,8]. Although this procedure leads

TABLE II

STABILITY[a] OF DIFFERENT FOLATE DERIVATIVES AFTER HEATING OR ACETONE TREATMENT

Experiments were carried out as described under Experimental.

Derivative	Stability (%) (± S.D., $n = 3$–5)		Difference[b]
	Heating	Acetone treatment	
PteGlu	93.6 ± 1.6	99.1 ± 2.2	$p \leqslant 0.001$
DHF	0	27.5 ± 5.4	$p \leqslant 0.001$
THF	95.1 ± 3.6	99.4 ± 2.9	n.s.
5-CH_3-THF	101.0 ± 1.6	98.9 ± 1.3	n.s.
5-CHO-THF	91.5 ± 1.8	102.9 ± 4.2	$p \leqslant 0.001$
MTX	91.7 ± 7.2	98.5 ± 0.9	n.s.
DCF	94.3 ± 4.6	90.0 ± 6.3	n.s.

[a] Stability is the amount of substrate found after carrying out the experiments compared with samples that had been assayed immediately.

[b] Statistical differences between the two procedures as judged by Student's *t*-test; n.s. = not significantly different.

to complete hydrolysis, it is not suitable for the analysis of folates. Because of the activity of other enzymes of folacin metabolism which are also liberated during the sample preparation, interconversions between several folates can be observed [19].

The second method for the hydrolysis of polyglutamates is to inactivate all cellular enzymes to fix the folate pattern and to add a purified PPH preparation [7,11,13,14,20]. This method minimizes the possible interconversions between different folacins described and was therefore chosen by us (Fig. 1).

Our comparative studies in which the pteroylpolyglutamates were hydrolysed by incubating the homogenized tissue for 60 min led to a different folate pattern compared with the use of a purified PPH (Table III). This emphasizes that the first technique produces artefacts. To prevent such faults the tissue must therefore be cut only coarsely and must be heated before homogenization. This step is necessary to inactivate the enzymes responsible for changes in the folate pattern and, further, enables the state of 'free folate' to be fixed. The differentiation between 'free folate', *i.e.*, the monoglutamates, and 'total folate', which includes mono- and polyglutamate forms, is of nutritional interest. The bioavailability of folacin monoglutamates is higher than that of the polyglutamates because the latter must be hydrolysed prior to intestinal absorption and they are therefore only partially utilized [27,28].

Pretests revealed that hydrolyis of polyglutamates failed when samples which had been extracted with acetone were incubated, even though the organic solvent had been removed under a stream of nitrogen. We assume that traces of acetone that remained in solution denatured the enzyme.

The experiments with PPH suspension revealed that complete hydrolysis of polyglutamates from cabbage and liver was achieved rapidly. After 30 min no further increase in monoglutamates could be measured. Additional studies in which solutions of several folacins were incubated with the PPH suspension showed that no con-

TABLE III

FOLATE CONTENT (±S.D.) AND FOLATE PATTERN OF RAT LIVER AS DETERMINED BY VARIOUS HPLC PROCEDURES WITH DIFFERENT DETECTION SYSTEMS

Where necessary the original data have been changed to nmol/g liver.

Detection after chromatography	THF (nmol/g)	%[a]	5-CH_3-THF (nmol/g)	%[a]	CHO-THF[b] (nmol/g)	%[a]	Total (nmol/g)	Ref.
Microbiological assay	11.46 ± 1.78	42	10.20 ± 1.71	39	5.64 ± 1.58	19	27.3	11
Microbiological assay	4.63 ± 1.39	33	5.12 ± 1.85	37	4.04 ± 1.39	30	13.8	14
UV	1.30 ± 0.39	22	3.53 ± 1.02	61	0.93 ± 0.22	17	5.8	20
UV	5.70 ± 1.00	36	7.34 ± 1.20	48	2.37 ± 0.70	16	15.4	29
Fluorimetry	9.64 ± 0.58	43	5.51 ± 1.65	25	7.10 ± 0.53	32	22.3	7
Fluorimetry	3.61 ± 0.24	29	7.69 ± 0.60	61	1.27 ± 0.24	10	12.6	8
Fluorimetry	3.15 ± 0.43	29	5.21 ± 0.47	49	2.40 ± 0.52	22	10.8	This work[c]
Fluorimetry	3.31 ± 0.37	30	6.24 ± 0.59	57	1.43 ± 0.33	13	11.0	This work[d]

[a] Percentage of total folate.

[b] CHO-THF is the sum of 10- and 5-CHO-THF.

[c] Using a purified PPH suspension to hydrolyse the pteroylpolyglutamates.

[d] Incubating the homogenized tissue for 60 min at 37°C for liberating the pteroylpolyglutamates by endogenous PPH.

version of the substrate to other metabolites occurred within 2 h, thus indicating that the suspension was free from any side activities which might have led to interconversion between several folates.

Analysis of rat liver folates

Fig. 3 shows a typical chromatogram of rat liver folates. Stop-flow spectra of the sample peaks recorded at the peak maxima were compared with those of standard folates and found to be identical, thus indicating the suitability of the analytical procedure. The small shoulder peak near THF could be discriminated by integration. Table III lists the folate contents determined by our procedure as shown in Fig. 1 in comparison with other data, indicating that our results are within the same range. The internal standards MTX and DCF, which were used to monitor the extraction and chromatography, could be recovered to the extent 70.5 ± 4.5% for MTX and 75.3 ± 4.2% for DCF. These correspond to the amounts of these two substances that were expected to be recovered: taking into account the mean extraction efficiency for folates under these conditions (77.3%, Table I) and the thermal destruction of DCF and MTX (Table II), it can be calculated that about 70.9% of MTX and 72.9% of DCF should be recovered by the analytical procedure.

Table IV shows the reproducibility of the overall method as determined by fivefold analysis of the same sample, demonstrating that the procedure shows good reproducibility. When interpreting these small variations it should not be overlooked that these discrepancies might even be due to an inhomogenity of the sample itself, five small pieces of liver having to be analysed separately.

The discrepancies of about 500% between different methods even with the same detection system (Table III) can be attributed to a certain extent to differences in

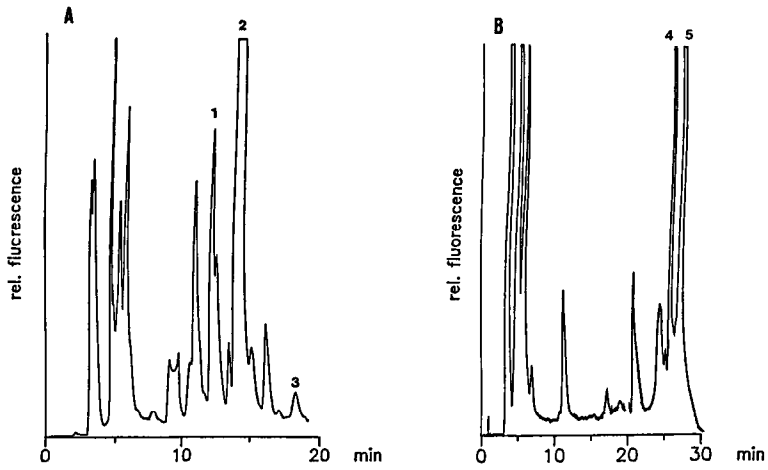

Fig. 3. Typical chromatogram of rat liver folates. Separation conditions as in Fig. 2. Detection: (A) native fluorescence of reduced folates with excitation at 295 nm and emission at 356 nm; (B) fluorescence (excitation at 365 nm, emission at 450 nm) of the products obtained by oxidation of several folates using post-column derivatization with 1% potassium peroxodisulphate. Reagent was pumped in the eluent stream leaving the first detector at a flow-rate of 0.3 ml/min. Peaks: 1 = THF; 2 = 5-CH_3-THF ; 3 = 5-CHO-THF; 4 = MTX; 5 = DCF.

TABLE IV

PRECISION OF THE WHOLE ANALYTICAL PROCEDURE AS DETERMINED BY FIVEFOLD ANALYSIS OF THE SAME LIVER SAMPLE

Extraction, hydrolysis of pteroylpolyglutamates and HPLC analysis were carried out with five small pieces obtained from one liver. Values given are the means ± S.D.

Derivative	Content (nmol/g liver)
THF	3.05 ± 0.26
5-CH_3-THF	5.35 ± 0.23
CHO-THF[a]	2.55 ± 0.17

[a] CHO-THF is the sum of 10- and 5-CHO-THF.

breed, age and nutritional state of the animals used [7]. However, it should be assumed that they are mostly caused by the analytical procedure itself. Duch *et al.* [20], for example, used a prolonged extraction and sample clean-up procedure which included cation-exchange chromatography, lyophilization and a storage of the samples in buffer of pH 8.3 at 4°C for 3 h, which explains the very low contents that they obtained.

Table III also shows that the folate patterns found by several authors are different. In this context it is interesting to consider the results published previously by Gounelle *et al.* [8], who found only 10% of the whole folate activity in the formyl derivatives. They used a technique to convert 10-formyl-THF to 5-formyl-THF, with subsequent measurement of the latter, and concluded that their results were due to incomplete conversion of the 10- to 5-formyl derivative. The technique of heat conversion that they used was first described by Gregory *et al.* [7], who reported a conversion rate of 99% within 1 h. Therefore, the objections raised by Gounelle *et al.* [8] do not seem to explain this result. On the other hand, the latter group hydrolysed the polyglutamates by incubating the homogenized tissue, which might have led to interconversions such as we observed when applying the same technique. This supports the view that only purified PPH suspensions should be used in folacin analysis.

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