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Comparison of a HPLC and radioprotein-binding assay for the determination of folates in milk and blood samples

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A high-performance liquid chromatographic method for the determination of folates in milk, whole blood and plasma following thermal extraction, enzymatic deconjugation and a clean-up step with a strong anion exchange column is described. The optimized and rapid HPLC method was carried out on a reverse phase C_{18} column where the native fluorescence was monitored at excitation and emission wavelengths of 310 and 352 nm, respectively. The method is sensitive with low detection limits for 5-CH_3THF, THF and 5-CHOTHF, below 3 pmol/injection. The intra-and interassay CV for the individual folate standards is in the range of 4.0 – 8.8%. 5-CH_3THF was the dominant form found in all samples. The mean ±SD, concentrations of total folate in milk (with and without conjugase treatment), whole blood and plasma were 45.6±4.6 ng/ml, 25.2±1.8 ng/ml, 239.9±36.5 nmol/litre, and 8.0±1.9 mmol/litre. Corresponding figures using a commercial protein-binding assay kit were 47.7±9.8 ng/ml, 79.6±8.8 ng/ml, 522.2±90.3 nmol/litre, and 10.7±3.8 mmol/litre, respectively. The average recovery of 5-CH_3THF, THF and 5-CHOTHF added to milk samples after heat extraction were 106, 70 and 0%, respectively. After deconjugation the recovery for 5-CH_3THF decreased while it increased for THF, suggesting that constituents in the biological matrix either degraded or transformed these folates into other forms.

INTRODUCTION

The folates represent a nutritionally important vitamin among the B-group. This vitamin is essential for single-carbon transfer reactions required in many metabolic pathways including the important nucleic acid biosynthesis as well as several amino acid interconversions, (Brody *et al.*, 1984).

Naturally occurring folates exist primarily as reduced one-carbon substituted forms of pteroylpolyglutamates, and have a varying number of glutamyl residues attached to the pteroyl group (Shane *et al.*, 1980). These vitamers may differ in their bioavailability (for overview see Halsted, 1990), and it is therefore desirable to quantify the individual forms. Analyses of native folates are complicated not only because the vitamin occurs in many different forms, but also because these folates are light-and heat-sensitive and are easily destroyed by oxidation (Brody *et al.*, 1984).

At present, the most widely used and accepted procedure is the microbiological assay using *Lactobacillus casei* (ATCC 7469) as test organism, where a growth response of the organism to the mixture of folates present are measured turbidometrically. This organism responds to most native folates, though with a decreasing response to an increasing number of glutamyl residues linked to the pteroyl group (for overview see Tamura, 1990). In order to measure the polyglutamated forms, these must be enzymatically deconjugated prior to analysis. In different studies concerning the procedure for deconjugation of food folates, the type of food, source of conjugase, pH and incubation time have varied and the optimal conditions have not yet been fully elucidated.

For clinical purposes, i.e. analysis of folates in blood and plasma, methods based on enzyme protein-binding assays (EPBA) and commercial radioprotein-binding assay kits (RPBA) have replaced the microbiological method. The extent to which these methods are also suitable for food analyses remains to be established. Their specificity for individual folate forms, 5-CH_3THF, THF and 5-CHOTHF, has been reported as being not uniform, and the response to conjugated folates is also unclear at present (Shane *et al.*, 1980).

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During the past few decades, chromatographic procedures using HPLC have become the most widely used technique in vitamin analyses of food.However, the low levels of folates found in foods, along with different vitamin forms have so far prevented the successful development of HPLC procedures for this vitamin. Moreover, most published methods present data using various folate standards (Reingold *et al.*, 1980), and only a few studies have reported on the analysis of native folates in foods or body fluids (Day & Gregory, 1981; van Niekerk, 1988; Müller, 1993*a*; Müller, 1993*b*).

The overall objective of this study was to evaluate a convenient, specific and rapid method for the determination of folates which would be suitable both for dietary and clinical samples, with the ultimate goal being to study the bioavailability of dietary folates. A reverse-phase HPLC technique was developed and compared with a protein-binding assay (RPBA, commercial kit) for folate analysis of cow milk, human whole blood and plasma.

MATERIALS AND METHODS

Chemicals

All chemicals were of *pro analysi* grade. Methanol and acetonitrile (Lichrosolv, gradient grade) were purchased from Merck (Darmstadt, Germany) and acetic acid (99–100%) was procured from Baker (Deventer, Holland). Water was obtained from a Milli-Q water purification system (Millipore, Stockholm).

5-Methyltetrahydrofolate (5-CH_3THF) (calcium salt) was obtained from Schirck's (Jona, Switzerland). Tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-CHOTHF) (calcium salt), pteroylmonoglutamic acid (PGA), sodium ascorbate and 2-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO).

For relative accuracy when analyzing dietary folates, standard solutions of folates with a concentration of 1 mg/ml were calibrated, according to the procedure of van den Berg *et al.* (1994), by dissolving the crystalline folate compound directly in 0.05-M sodium borate with 0.4% (v/v) 2-mercaptoethanol to mark (e.g. 10 ml). Aliquots (100 μl) of these solutions were taken for dilution (e.g. 10 ml of phosphate buffer, pH 7) and spectrophotometric determination of the true concentration. The specific wavelength for maximum absorption was found by UV scanning. The relative absorbance ratio was calculated according to Lambert Beer's law using the molar extinction ($\times 10^{-3}$) according to Blakely (1969), see Table 1. The remainder of each solution was immediately diluted in 1% (w/v) sodium ascorbate up to mark (usually 10 ml). These solutions were used directly or stored at −20°C.

Table 1. Spectral data for folates on maximum absorbances and the molar extinction

Compound	Max (nm)	(10^3)
PGA	287	27.6
5-CH_3THF	290	31.7
THF	297	29.1
5-CHOTHF	285	37.2

Samples

Commercial (low-fat, 0.5%) milk ($n = 10$) was purchased from a local store. Venous blood from five healthy volunteers was collected into tubes containing EDTA as an anticoagulant. Plasma was separated from the whole blood fraction by spinning for 10 minutes at 4500 rpm at 4°C. The volunteers involved were two men and three women aged 27–55 years.

Extraction and deconjugation of milk, whole blood and plasma for HPLC analysis

Commercial milk samples were placed undiluted in screw-cap polypropylene centrifuge tubes (approx. 50 ml) to which sodium ascorbate was added, yielding 1%. All milk samples, which were carefully protected from light, were placed in a boiling water bath for 15 min, and then in an ice bath where they were left to cool to room temperature. In order to be measured, the polyglutamate forms in milk have to be enzymatically deconjugated. Human plasma deconjugation was used according to the conditions employed in a recent intercomparison of methods study for folates (Vahteristo *et al.*, 1995). Human plasma (5 g) was purchased from Sigma Chemical Co. (St. Louis, MO) and diluted in 20 ml of 100 mM 2-mercaptoethanol under gentle agitation.

Three ml of the milk supernatant from each tube was mixed with 0.4 ml of the human plasma conjugase and extraction buffer (0.1-M phosphate buffer, pH 6.0, containing 0.5% sodium ascorbate). Incubation of the milk samples took place in a 37°C water bath for 1 h, carefully protected from light. An enzyme blank solution and a milk blank solution were also included.

Light-protected whole blood and plasma samples (1.5 ml) were diluted (x2) in 1% sodium ascorbate solution. One ml of the diluted whole blood samples was mixed in 7 ml of water to ensure effective haemolysis. Thereafter 2 ml of 0.25-M potassium phosphate buffer (pH 6.1, containing 0.3% sodium ascorbate) was added (Hansen, 1964). Incubation of the whole blood samples, diluted in 1% sodium ascorbate and yielding a total dilution of 1:40, was performed at 37°C for 90 min to permit deconjugation of erythrocyte folate polyglutamates through the action of endogenous plasma conjugase activity. Plasma was not subjected to deconjugation.

Preparative chromatography

After spinning the samples (15 000 rpm for 10 min), the supernatant was loaded onto a disposable strong anion-exchange column (Sorbent AB) that had been

pretreated with one column-volume each of methanol and water (Schieffer *et al.*, 1984). A vacuum manifold (Vac Elute, Analytichem International) capable of holding up to 10 disposable columns was used to greatly facilitate and enhance the elution. The solution was pulled through the column under vacuum and the eluate was discarded. After washing with two column volumes of water, the folates were eluted with 1 ml of a 10% sodium chloride solution containing freshly added 1% sodium ascorbate. Prior to the HPLC analysis, the eluate was filtered through a Millipore filter (0.45 μm).

HPLC system

The chromatographic analysis was performed by using a commercial HPLC system (Varian Chromatography Systems, Walnut Creek, CA, model 5000) consisting of a single-piston, reciprocating pump, where a proportioned delivery of two solvents can be achieved and an autosampler (Waters Intelligent Sample Processor, WISP™, model 712). Measured aliquots of 1–2000 μl, adjustable in 1 μl increments, can be programmed for up to 48 sample vials. The injection volume was 100 μl and the flow rate 0.4 ml/min. A 4.0×30-mm guard column containing a cartridge packed with 5 μ of octadecylsilica (E. Merck, Darmstadt, Germany) was installed before the reverse phase LiChrospher 100 RP-18 analytical column (5-μm octadecylsilica, 250×4 mm, E. Merck, Darmstadt, Germany). The column temperature, 27°C, was kept constant throughout the whole experiment by means of a column heating system. The native fluorescence of the folates was monitored with a spectrofluorophotometer (Shimadzu, RF 540) equipped with a LC flow cell (20 μl) volume. The excitation was set at 310 nm and the emission wavelength at 352 nm. The excitation and emission slit widths were set at 5 and 40 nm, respectively. The mobile phase was 8% acetonitrile in acetic acid, pH 2.3. The output was integrated and recorded with a Shimadzu data processor, chromatopac C-R3A, and a Radiomatic Flo-one/Beta, A500 series, version 1.5 for Windows software. Peak area versus the amount of compound injected on the column was used for the calculations. A standard mixture was rerun periodically during the course of analysis.

Radioprotein-binding assay (RPBA)

This analysis was performed using a commercial RPBA kit (Kodak, former Amersham). Dilution of whole blood and plasma was done accordingly to the manufacturer, yielding a working pH of 9.2 which is the optimum pH of the standard solutions in the kit. Milk was diluted using a zero standard, provided by the kit, with a composition similar to that of human plasma, yielding a total dilution of 1:10. PGA in six concentrations (0–20 ng/ml) was used as a calibrant and was supplied in the kit. Calculations of sample folate concentration and plotting of standard curves was performed using a RiaCalc Ltd program supplied by Pharmacia (Uppsala, Sweden). A γ-scintillation counter (1282, LKB Wallac, Sweden) was used for measurements of the relative radioactivity. The pH of the diluted samples in the working solution was carefully controlled to avoid large variations in pH.

The responses of calibrated standard solutions of PGA, 5-CH_3THF, THF and 5-CHOTHF were also investigated. Prior to analysis, standard solutions of these four folate forms were diluted in 1% (w/v) sodium ascorbate, to the same six concentrations as the standard concentrations of the kit (0–20 ng/ml).

RESULTS

Sample preparation

The first step in isolating dietary folates in foods and body fluids usually includes heat-extraction by boiling or autoclaving in the presence of ascorbic acid. This is done in order both to protect the labile reduced folate forms from being oxidized and destroyed by endogenous enzymes, and to liberate protein-bound folates.

In addition, a clean-up procedure was necessary in order to obtain good separation and identification of the native folates on HPLC. All three forms of folates were completely recovered during the clean-up step

Table 2. Recoveries (%) of 5-CH_3THF, THF and 5-CHOTHF mixed in an aqueous solution containing 1% sodium ascorbate, *per se* and added to plasma, milk and blood samples. All samples were subjected to a strong anion exchange column prior to the HPLC analysis

	No heat extraction	After heat extraction	After deconjugation
Standard mix	^{1}101 ± 3	^{1}105 ± 5	
	^{2}99 ± 2	^{2}91 ± 5	
	^{3}95 ± 2	^{3}93 ± 10	
Plasma	^{1}55 ± 2		
	^{2}67 ± 9		
	^{3}53 ± 11		
Milk		^{1}106 ± 12	^{1}66 ± 8
		^{2}70 ± 8	^{2}165 ± 33
		^{3}0	^{3}0
Blood			^{1}84 ± 5
			^{2}143 ± 37
			^{3}70 ± 5

Mean recoveries ($n=5$) ± SD; ^{1}5-CH_3THF; ^{2}THF; ^{3}5-CHOTHF The mean recoveries ($n=5$) ± SD for an aqueous solution of 5-CH_3THF, THF and 5-CHOTHF, containing 1% sodium ascorbate were 101 ± 3, 99 ± 2 and 95% ± 2%, respectively. The corresponding mean recoveries ($n=5$) ± SD of spiked plasma samples subjected neither to heating nor to deconjugation were 55 ± 2, 67 ± 9 and 53% ± 11%, respectively. The mean recoveries ($n=5$) ± SD of the combined heat extraction and clean-up procedures for an aqueous solution of 5-CH_3THF, THF and 5-CHOTHF, containing 1% sodium ascorbate were 105 ± 5, 91 ± 5 and 93% ± 10%, respectively. Corresponding figures for spiked milk samples were 106 ± 12, 70 ± 8 and 0%, respectively. Heat extracted spiked milk samples and non-heat treated whole blood samples, subjected to deconjugation showed corresponding values of 66 ± 8, 165 ± 33, 0 and 84 ± 5, 143 ± 37, 70% ± 5%, respectively.

using strong anion exchange columns which is displayed in Table 2.

HPLC separation and analysis

Good separation of the three individual folate forms used in the standard mixture was obtained (Fig. 1). Standard curves were obtained for all three compounds and showed a linearity, over the following ranges which permitted the use of single level calibration standards: THF, 1.4–28.7 pmol (0.74–17.8 ng/injection, $r = 0.997$), 5-CH_3THF, 0.08–35.8 pmol (0.04–15.5 ng/injection, $r = 0.999$), and 5-CHOTHF, 2.9–116.1 pmol (1.49–59.4 ng/injection, $r = 0.993$).

The detection limits for 5-CH_3THF, THF and 5-CHOTHF, defined as the concentration yielding a signal:noise ratio of 3, were 0.08, 1.4, 2.9 pmol/100 μl injection (0.04, 0.74, 1.49 ng/100 μl injection). The interassay CVs for the individual folates were 4.0, 4.7 and 8.8%, respectively. The repeatability of 10 injections (intra-assay) showed a CV of less than 4% for the individual folate standard solutions, demonstrating good precision for the method. Since the HPLC was equipped with an autosampler, the stability of the reference mixture was checked. All three folate forms were reasonably stable up to 24 h in 1% sodium ascorbate at room temperature as illustrated in Fig. 2.

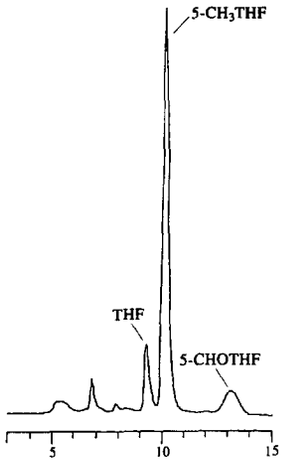

Fig. 1. HPLC chromatogram of a standard mixture of THF, 5-CH_3THF and 5-CHOTHF.

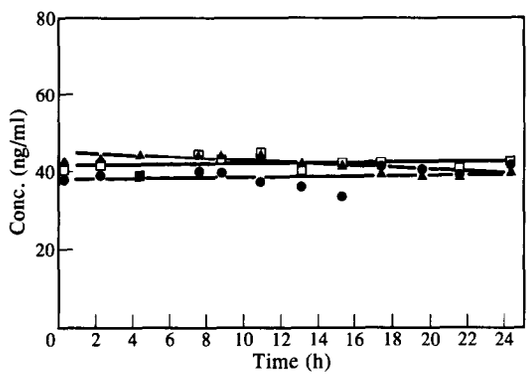

Fig. 2. Stability of THF (●), 5-CH_3THF (▲) and 5-CHOTHF (□) during HPLC analysis using an autosampler for 24 h.

The 5-CH_3THF was the dominating form detected in ten commercial (low-fat, 0.5%) milk samples, five whole blood and five plasma samples. Representative chromatograms of the analysis of hydrolyzed and non-hydrolyzed commercial low-fat milk, whole blood and plasma are shown in Fig. 3(a–d).

The mean, ±SD, concentrations of 5-CH_3THF in milk (with and without conjugase treatment), whole blood and plasma were 45.6±4.6 ng/ml ($n = 10$), 22.6±1.8 ng/ml ($n = 10$), 239.9±36.5 nmol/litre ($n = 5$), and 8.3±1.9 mmol/litre, ($n = 5$), respectively. The mean ±SD concentrations of THF in non-treated milk, whole blood and plasma were 2.6±1.3 ng/ml ($n = 10$), 54.7±21.7 nmol ($n = 5$), and 0.4±0.1 mmol/litre ($n = 5$), respectively. However, THF was not detected in conjugase-treated milk and no 5-CHOTHF was detected in any of the samples.

The conjugase treatment increased the milk folate values, but only for 5-CH_3THF. Enzyme blanks showed no interfering peaks. Data on folate concentrations of hydrolyzed and non-hydrolyzed samples obtained by HPLC analysis and RPBA are presented in Table 3.

RPBA

According to the RPBA method, the mean ±SD concentrations of total folate in milk (with and without

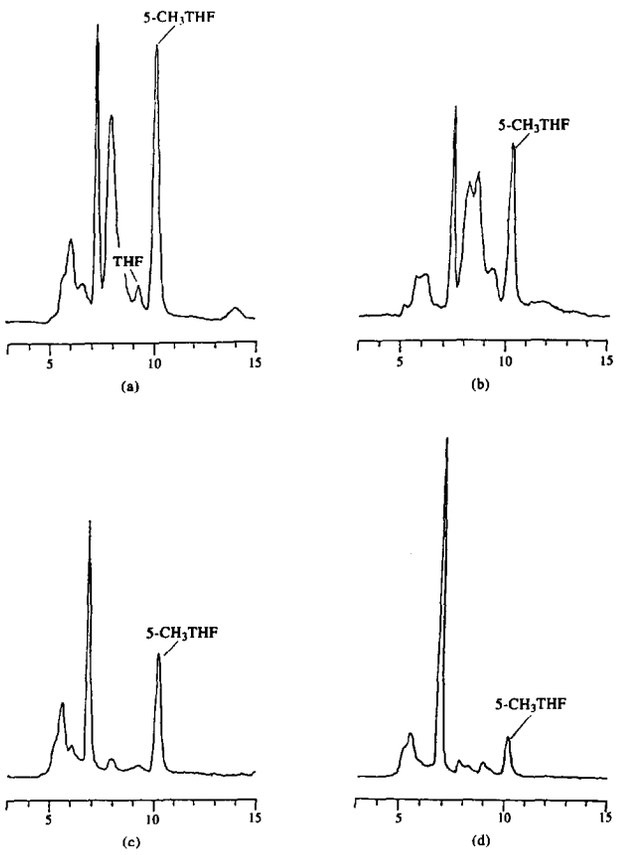

Fig. 3. (a) Representative HPLC chromatogram of non-conjugated milk folates. (b) Representative HPLC chromatogram of conjugated folates, diluted 10 times compared to the milk sample, shown in (a). (c) Typical chromatogram of human plasma. (d) Typical chromatogram of human whole blood.

Table 3. Comparison between HPLC and RPBA, analyzing folates in low fat milk, human plasma and whole blood. The RPBA estimates have been calculated using the PGA (kit) calibrant and the in-house 5-CH_3THF and PGA calibrants

Sample		HPLC		RPBA		
	n	5-CH_3THF	THF	PGA_{kit}	PGA	5-CH_3THF
Low fat milk[a] (ng/mlitre)	10	22.6 ± 1.8[b]	2.6 ± 1.3[b]	79.6 ± 8.8[b]	74.0 ± 8.2[b]	51.0 ± 5.6[b]
	10	45.6 ± 4.6[c]	nd[c]	47.7 ± 9.8[c]	44.3 ± 9.1[c]	30.6 ± 6.3[c]
Plasma (nmol/litre)	5	8.0 ± 1.9	0.4 ± 0.1	10.7 ± 3.8	10.0 ± 3.5	6.9 ± 2.4
Whole blood (nmol/litre)	5	239.9 ± 36.5	54.7 ± 21.7	522.2 ± 90.3	485.5 ± 84.0	334.6 ± 57.9

Mean ± SD.
[a]0.5% fat.
[b]Before conjugase treatment.
[c]After conjugase treatment.

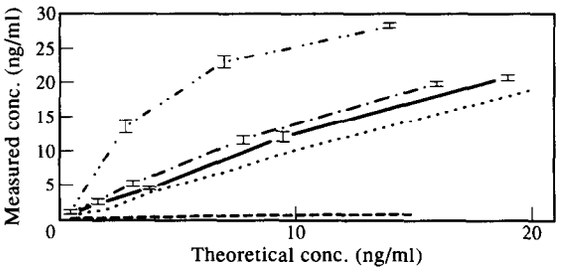

Fig. 4. RPBA response of THF (-···), 5-CH_3THF (-··), 5-CHPTHF (—), PGA (—) and PGA:kit (····).

conjugase treatment), whole blood and plasma were 47.7 ± 9.8 ng/ml ($n = 10$), 79.6 ± 8.8 ng/ml ($n = 10$), 522.2 ± 90.3 nmol ($n = 5$), and 10.7 ± 3.8 mmol/litre ($n = 5$), respectively (Table 3).

The RPBA responses of 5-CH_3THF, THF and 5-CHOTHF at the actual working pH of 9.2, which is the optimal pH in the RPBA kit with regard to the standard calibrants included, were investigated. The results are displayed in Fig. 4 and show a marked overestimation (2- to 3-fold) for THF and a certain overestimation also for 5-CH_3THF and our own PGA standard compared to the kit standard (PGA). In contrast, no response was seen at all for 5-CHOTHF.

DISCUSSION

HPLC

An optimized, rapid and convenient HPLC assay on the determination of dietary folates in milk and clinical samples (plasma and whole blood) is presented. Samples were heat-extracted in a boiling water-bath in the presence of 1% sodium ascorbate to protect folates from oxidative degradation. Since naturally occurring folates exist primarily as pteroylpolyglutamates, with a varying number of glutamyl residues attached to the pteroyl group, they have to be enzymatically deconjugated prior to analysis. Human plasma deconjugation of milk samples was used according to the conditions employed in a recent intercomparison of methods study for folates (Vahteristo *et al.*, 1995). Prior to HPLC analysis, a clean-up procedure using a strong anion-exchange column was conducted according to Schieffer *et al.* (1984). The conditions for the HPLC separation were mainly derived from Day & Gregory (1981), although there were some modifications. Both methods involve reverse phase chromatography, an isocratic elution system of 10–11% acetonitrile, the principle of low-pH (2.3), converting folates into non-polar forms without adding ion-pairing agents such as tetrabutylammonium phosphate and monitoring native fluorescence at similar excitation and emission wavelengths. Our method, however, used acetic acid instead of 0.33-M sodium phosphate buffer, a single column system and another flow rate yielding much shorter retention times (all below 20 min) compared to the 90 min required in the study by Day & Gregory (1981).

The validity of this HPLC procedure was checked using a standard mixture of 5-CH_3THF, THF and 5-CHOTHF, which showed base-line separation. The detection limits for these folate derivatives were in the picomole range and demonstrated high sensitivity, sufficient for detecting physiologically relevant concentrations of folates in milk, whole blood and plasma. Linearity and precision data were also satisfactory. Recovery of the standard mixture was followed in each separate step and yielded over 90% following heat-extraction, conjugase treatment, clean-up and HPLC injection.

Spiking of milk, whole blood and plasma samples was conducted in order to support the identity of the peaks and also to check the recovery. Spiking heat extracted, unconjugated milk with 5-CH_3THF yielded overall recoveries close to that of the standard mixture in water (~100%). However, spiking milk samples with THF and 5-CHOTHF resulted in considerable losses during heat extraction. THF is known to be very unstable (Brody *et al.*, 1984), which probably explains its low recovery. However, a great increase of THF recovery was seen post deconjugation, suggesting that constituents in the biological matrix either degraded or transformed these folate forms into other forms, THF being presumably one of them. It should be noted, that the standard mixture of 5-CH_3THF, THF and 5-CHOTHF in aqueous solutions containing 1% sodium ascorbate acid was shown to be stable up to 24 h during the HPLC analysis (Fig. 2). In addition, the autosampler was loaded with samples of standards and biological samples containing

1% freshly-added ascorbic acid which ran only for 12-h intervals in order to prevent degradation. The recoveries of unconjugated milk samples spiked with 5-CH_3THF, THF and 5-CHOTHF obtained in the present study are similar to those reported earlier by Gregory *et al.* (1984) and Holt *et al.* (1988).

The low recoveries of spiked plasma and wholeblood samples, analyzed by HPLC was unexpected. According to van den Berg *et al.* (1994), low recovery (40%) was also seen in sera spiked with 5-CH_3THF using the microbiological assay, while the RPBA yielded a recovery of 100–150%. However, addition of human plasma conjugase might presumably lower the affinity for folates during the clean-up procedure using strong anion-exchange columns. The same explanation could also apply to the low recoveries seen for spiked human plasma samples. These results emphasize the need of spiking samples routinely, in order to be aware of probable losses obtained during sample preparation prior the HPLC analysis of folates. However, no correction for recoveries was included in the folate values presented in Table 3.

Cow milk samples were shown to contain almost solely 5-CH_3THF in mono-and polyglutamic forms. A small amount of THF was demonstrated in non-treated cow milk samples, but not in the conjugase-treated samples. No 5-CHOTHF was present either in unhydrolyzed or hydrolyzed cow milk samples. The conjugase treatment increased only the fraction of 5-CH_3THF. This is in agreement with Selhub (1989), who stated that polyglutamyl folates in milk are exclusively 5-CH_3THF derivatives. It also corresponds with earlier findings (Karlin, 1969; Shin *et al.*, 1975; Dong & Oace, 1975; Gregory *et al.*, 1984) showing that 90–95% of the folates in cow milk represent 5-CH_3THF. According to Selhub (1989), cow milk consisted of monoglutamyl (73%) and polyglutamyl derivatives. The polyglutamyl folates were exclusively 5-CH_3THF, whereas 50% of the monoglutamylfolates were 5-CH_3THF and 23% were pteroylglutamate folates. Using microbiological assay, Karlin (1969) reported 90–95% 5-CH_3THF in cow milk, 60% of which was comprised of mono-and 40% polyglutamates. Our data indicated around 50% mono- and 50% polyglutamates. The total amount of folates in milk after conjugase treatment in the present study showed a total folate value of 46.6 ± 4.6 ng/ml. These values are close to the values reported in the literature based on microbiological assays (Karlin, 1969; Harzer & Haschke, 1989).

The plasma and whole blood mean folate concentrations, based on HPLC analysis, fall within the ranges found previously in clinical studies for healthy humans (Kohashi *et al.*, 1986; Leeming *et al.*, 1990). In accordance with earlier reports, plasma was shown to contain only 5-CH_3THF, which separated well into a nice clean peak. Whole blood, on the other hand separated into two fractions yielding one major peak of 5-CH_3THF, (239.9 ± 36.5 nmol/litre) and one minor peak of THF (54.7 ± 21.7 nmol/litre). Red cells and other mammalian tissues are known to contain mainly polyglutamate folates (Eto & Krumdieck, 1981), which appear to have been conjugated by the endogenous conjugase present in whole blood. According to Leeming *et al.* (1990), other intracellular folate polyglutamates are mainly THF and 10-CHOTHF where 10-CHOTHF is utilized in the novo biosynthesis of the purine ring. Monoglutamates are principally represented in plasma where 5-CH_3THF is the dominant form (Scott & Weir, 1976).

Several HPLC studies have been reported on the separation of standard solutions of different folate forms (van Niekerk, 1988), whereas only a few methods deal with analysis of dietary folates in bovine milk, human whole blood and human plasma. Reingold *et al.* (1980) analyzed folates in human milk. Gregory *et al.* (1984), Holt *et al.* (1988) and Müller (1993b) measured folates in bovine milk. Ristow *et al.* (1982) compared HPLC methods measuring 5-CH_3THF and folic acid in various lactose–casein liquid model systems, whereas Day & Gregory (1981) and Schieffer *et al.* (1984) measured folate content in infant formula.

Fluorimetric detection is considered superior to UV detection since it is highly sensitive in acidic media and the problem of interference from nucleotides that strongly absorb UV light is reduced. For satisfactory detection of native fluorescence of the reduced folates, high sensitivity is required (Gregory *et al.*, 1984). Moreover, the analysis has drawbacks due to the instability of THF and the relatively weak fluorescence of 5-CHOTHF which could limit its applicability for quantifying folates in other food systems and tissues. The forefront of detection sensitivity may be represented by electrochemical detection with amperometric electrodes, which have minimally detectable levels in fmol/injection (Kohashi *et al.*, 1986). However, for uniform electrochemical detection of all folates, a relatively high potential (approximately +1 V) must be applied (Gregory, 1989). Due to this high oxidation potential, considerable background interference occurs which to a large extent appears to be ascorbic acid and 2-mercaptoethanol (Holt *et al.*, 1988). The electrochemical detection method has been successfully employed for the detection of 5-CH_3THF in plasma (Kohashi *et al.*, 1986; Lucock *et al.*, 1989). Electrochemical detection has also been used to measure native 5-CH_3THF in human cerebrospinal fluid (Lucock *et al.*, 1993) and liver homogenate (Lucock *et al.*, 1994), in the latter case as a dynamic measure of metabolism.

RPBA

Radioassays are based on the competition between the substance to be measured and a fixed quantity of radioactively labelled tracer for a limited number of specific binding sites. The sample containing unknown folates competes with ^{125}I-labelled folic acid (PGA) as the tracer for sites on folate-binding proteins derived from bovine milk. The method has been used routinely for clinical purposes during the past two decades, e.g. for analysis of folates in plasma and whole blood. The

RPBA assay does not discriminate between different forms of folates and it has been shown that pH is very critical in terms of the response. Givas & Gutcho (1975) found equal affinity for PGA and 5-CH_3THF at pH 9.3, while more recent studies by van den Berg & Floor (1990) found equality in affinities between these two folate forms at pH 8.9. The Kodak kit used in the present study showed similar responses for PGA and 5-CH_3THF at a pH close to 9. The pH-measurements of the calibrants supplied in the kit, commercial serum controls, milk diluted in zero standard, whole blood diluted in zero standard and whole blood diluted in 1% sodium ascorbate according to the manufacturer, showed a mean pH of 9.23 ± 0.01 ($n=11$), 9.20 ± 0.02 ($n=6$), 9.23 ± 0.01 ($n=2$), 9.20 ± 0.02 ($n=5$), and 9.24 ± 0.01 ($n=9$), respectively. The plasma folate values reported in this study using RPBA were slightly higher than those obtained with HPLC. The HPLC profile indicated only 5-CH_3THF, and the use of PGA rather than 5-CH_3THF as standard calibrant in the RPBA assay could be the reason for overestimation. For instance, when giving the folate concentrations in nmol/litre, an overestimation takes place, since the calculation is based on the molar mass of PGA (441.4 g/mol) rather than 5-CH_3THF (497.7 g/mol). Moreover, when checking the response of the RPBA kit towards 5-CH_3THF we found a certain overestimation compared to PGA, probably because our figures were corrected for purity. The HPLC analysis of whole blood indicated the presence of around 85% 5-CH_3THF and 15% THF. The sum of these two forms, however, was much lower than the values obtained in the RPBA assay. This could only be partly accounted for by those explanations regarding plasma, e.g. different calibrants. The major explanation for the discrepancy could be caused by the low recovery of spiking whole blood samples with folates. The same explanation could be used to explain the discrepancy of plasma folates. Thirdly, this discrepancy could be due to the overestimation by the RPBA kit of especially THF but also 5-CH_3THF which is demonstrated in Fig. 4.

There were two reasons the RPBA was used to compare the folate analysis. First, the assay is based on binding to a specific folate-binding protein obtained from bovine milk. In cow milk more than 90% of the folates are bound to such proteins (Henderson, 1990). Another reason the RPBA assay can be used for milk samples is that these samples contain almost exclusively 5-CH_3THF, which is known to show a good response in the RPBA assay. The comparison made in the present study between HPLC and RPBA values indicated similar folate values of around 45–50 ng/ml for both methods following conjugase treatment. However, the RPBA method showed values close to 80 ng/ml when the analysis was done without conjugase treatment. This higher value is in accord with one earlier study (Andersson & Öste, 1992) where RPBA and a microbiological assay were compared for the analysis of milk samples. In this study milk folate values in the range of 50–100 ng/ml were reported without using conjugase, and the authors stated that the RPBA assay responded equally well towards mono- and polyglutamic folates in milk samples. In our study the conjugase treatment of milk samples reduced the folate values when measured by RPBA. Different conjugase sources were used in these two studies, but it should be emphasized that the response of the RPBA kit towards conjugated folates needs further investigation. Another drawback to using the RPBA assay on dietary samples other than those consisting mainly of methylated folates is that the response of different folate forms has not been thoroughly investigated. It is known, however, that formylated folate, e.g. 5-CHOTHF, shows little if any response (Shane *et al.*, 1980 and present study). In addition, we also demonstrated the RPBA-method to markedly overestimate THF. A higher affinity, i.e. a more rapid binding of THF to RPBA compared to PGA or 5-CH_3THF could be an explanation. This overestimation of THF has not been reported previously but is an important observation considering the possibility that THF could be either produced or degraded from other folate forms during the sample preparation and analyses.

In conclusion, a rapid and convenient HPLC method for analysis of folates especially methylated folates in clinical as well as dietary samples is presented. Identification of the folate forms in milk is based on similarities in retention time of spiked and unspiked milk samples. This is of course only indirect evidence for purposes of identification. Nevertheless it was the only possible alternative in the present study, since the amounts analyzed were too small to be detected by UV and compared with the reference compounds with respect to spectral similarities, e.g. by using diode array detection. More work, consequently remains to be done on the reliability of the method when used for other folate forms. Another uncertainty this method has in common with all folate assay methods involves the hydrolysis of polyglutamate folates. No standardized procedure for this step has yet been adopted, and the conjugase preparations differ in sources, activities, optimal pH and hydrolysis products.

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