Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 788 (2003) 187–191
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Measurements of sub-nanomolar concentrations of unmetabolised folic acid in serum

Mary Rose Sweeney[a,1], Joseph McPartlin[a,*], Donald George Weir[a], John Martin Scott[b]

[a]*Department of Clinical Medicine, University of Dublin, Trinity College, Dublin, Ireland*
[b]*Department of Biochemistry, University of Dublin, Trinity College, Dublin, Ireland*

Received 21 May 2001; received in revised form 11 November 2002; accepted 5 December 2002

Abstract

We describe a combined HPLC/microbiological assay procedure for the sub-nanomolar analysis of unmetabolised folic acid (pteroylglutamate) in human serum. This metabolically unaltered form of the vitamin arises following the consumption of folic acid either in supplemental form or in fortified foods. Following HPLC separation of folic acid from other folate derivatives the folic acid fraction was concentrated by C_{18} Sep-Pak cartridges and assayed by *Lactobacillus casei* microbiological assay. The present assay allows the quantitation and kinetic analysis of the effects of consumption of folic acid.

Keywords: Folic acid

1. Introduction

In 1991 the Medical Research Council Vitamin study [1] showed conclusively that peri-conceptional folic acid supplementation would prevent 72% of NTDs. Folic acid fortification of grain, estimated to supply on average an additional 100 μg daily, has been introduced on a mandatory basis in the United States since 1998 and recent evidence shows that this measure has resulted in a 19% reduction in neural tube defects [2]. Many countries have begun procedures to follow the example of the US in fortifying flour based products.

Simulating fortification conditions, Kelly et al. [3] demonstrated the appearance of unmetabolised folic acid in the circulation after consumption of physiological doses of folic acid. While folic acid is not thought to be toxic, it may however contribute to the potential masking of pernicious anaemia in the elderly. Furthermore, there is the potential for folic acid to interfere with anticonvulsive therapy or cancer treatment with antifolates [4]. Apart from this, an assay for serum folic acid may be necessary to survey the variation in magnitude and kinetics of serum folic acid response to physiological doses of folic acid. While population variation would be expected to occur naturally, additional factors such

*Corresponding author. Sir Patrick Duns Vitamin Research Laboratory, Central Pathology Laboratory, St. James's Hospital, James's Street, Dublin 8, Ireland. Tel.: +353-1-608-2113; fax: +353-1-608-3551.
E-mail address: jmcprtln@tcd.ie (J. McPartlin).
[1]Present address: Dr. Mary Rose Sweeney, SurGen, Royal College of Surgeons, 123 St. Stephens's Green, Dublin 2, Ireland.

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(02)01010-3

as the effects of the practice of ''overage'' during food preparation may require monitoring.

Recent assay methods of free folic acid in blood using LC–mass spectrometry techniques demonstrate the potential for greater throughput and sensitivity [5] for the current problem, though the expense of such detection methods may prohibit their widespread use. In this paper we set out to improve the lower limit of quantitation of the methodology described by Kelly et al. [6]. This improved methodology involves the fractionation of unmetabolised folic acid in serum from other folate derivatives using high-performance liquid chromatography, followed by solid-phase concentration prior to assay by *Lactobacillus casei* (*L. casei*) microbiological assay [7].

2. Experimental

2.1. Sample collection and preparation

Whole blood was collected, allowed to clot and centrifuged for serum preparation. Tritiated folic acid [3′, 5′, 7′, 9′-^{3}H] Folic acid (^{3}H-Folic acid) (TRK212, 46 Ci/mmol, Amersham International, Bucks., UK) was purified routinely by isocratic HPLC (elution time 12–14 min) and 1.0×10^{4} dpm of ^{3}H-folic acid was added as internal standard to 500 μl of freshly prepared serum in 1.5 ml microfuge tubes. The serum samples were deproteinized by the addition of 50 μl of perchloric acid (60% v/v). Samples were then vortexed, frozen, thawed and the supernatant was removed. KOH (6 *M*) was added to the supernatant to bring the samples to pH 7.0.

2.2. HPLC separation

For each sample, 200 μl were injected manually onto a 100×8 mm C_{18} μBondapak (Radial Pak, Waters-Millipore, Milford, MA) cartridge column which was contained in a Z-Module (Waters-Millipore). The column was eluted isocratically in mobile phase consisting of sodium citrate disodium hydrogen orthophosphate buffer: (0.1 *M*, pH 4.0), acetic acid (1% v/v) and methanol (in the ratio of 43:42:15 respectively) at 3 ml/min for 20 min. One ml was taken from each of 6 fractions in the area of the folic acid retention time (12 min) [6] and added to 5 ml of Ecolite scintillation fluid (ICN, Costa Mesa, CA). Each sample was counted by scintillation counting to identify the radioactive peak. The peak occurred over 2 fractions. The remaining 2 ml from each of the two fractions containing the peak were then pooled to give a total volume of 4 ml.

2.3. Solid phase extraction by C_{18} SepPak

Four ml of citrate phosphate buffer (0.1 *M*, pH 4.0) was added to the 4 ml pooled folic acid peak to dilute the methanol in the mobile phase to prevent auto-elution of the folic acid from the C_{18} matrix. A Waters C_{18} SepPak was activated with 5 ml methanol followed by 5 ml sodium citrate phosphate buffer. The 8 ml sample was applied slowly to the SepPak, the retained folic acid being eluted at 1 ml/min with methanol and collected in a 1.5 ml centrifuge tube. The methanol was evaporated using a SpeedVac pump (Savant Instruments, Farmingdale, New York). The folic acid solute was re-suspended in 1.150 ml of sodium ascorbate (0.5%), 500 μl of which was assayed by scintillation counting to estimate recovery. Recovery was found to be 75–85% ($n=19$) of the amount injected onto the SepPak, corresponding to 4–5% of the original radiolabel added. A simple flow diagram (Table 1) illustrates the approximate recovery of the internal radiolabel standard at each step of sample preparation.

2.4. Microbiological assay for serum folic acid

L. casei microbiological assay for folic acid was performed on the remainder of the sample. A standard curve was constructed in the range of 0.625–12.5 pg/100 μl. Briefly, 100 μl of each re-suspended sample were plated out in triplicate onto a 96 well micro-titre plate, together with 200 μl of *L. casei*-containing growth medium. The plates were sealed with a plate sealer, incubated at 37 °C for 42 h. The samples were read on a Titertek Multiscan Plus (Labsystems. Helsinki, Finland) at a wavelength of 590 nm.

Table 1
Flow diagram of radiolabel internal standard throughout sample preparation and assay

Sample preparation	Approximate radiolabel recovery
1) Add ^{3}H to serum (500 μl)	100%
2) Deproteinise serum	80%
3) Apply 200 μl (40%) onto HPLC	32%
4) Assay of radiolabelled fractions (33% from each)	15%
5) Sep-Pak concentration	10%
6) Final extract	5%

2.5. Comparison of limit of quantitation of current versus previous method

Male and female subjects aged 20–30 years (n= 19) were recruited. All were found to be folate replete as determined by total serum and red cell folate status at recruitment. Pre-prandial serum from each individual was assayed to establish non-specific background (sample A). This was subtracted from the subsequent post-prandial values. Subjects were administered 1 slice of bread fortified with 200 μg folic acid. Serum samples were collected 1 (sample B) and 2 (samples C) h post-prandially. A second slice of bread fortified with 200 μg was then administered to the same subjects 2 h after sample C. Serum was again collected 1 (samples D) and 2 h (samples E) post-prandially. All serum samples were assayed by the original and current method.

3. Results

3.1. Calculation of serum folic acid ng/ml [FA]s

$$[\text{FA}]\text{s} = [(c \times 5)/\%\ \text{recovery}] \times 2$$

where c is the value obtained from the microbiological assay standard curve. The factor of 5 adjusts the microbiological assay volume from 100 μl, i.e. the assay volume, to 500 μl, corresponding to the volume counted for radioactivity. The % recovery refers to the radioactivity recovered in the 500 μl re-suspended sample extract. The factor 2 adjusts the final results to ng/ml.

3.2. Limit of quantitation of the assay

This refers to the lowest quantifiable limit of the assay. The lowest standard curve value of the microtitre plate assay is 0.625 pg/100 μl. Hence the lower limit of detection of this assay when applied to the formula above is

$$\begin{aligned}[\text{FA}]\text{s} &= [(0.625 \times 5)/0.05] \times 2 \\ &= 31.25\ \text{pg}/100\ \mu\text{l} \\ &= 312.5\ \text{pg/ml} \\ &= 0.3125\ \text{ng/ml}\end{aligned}$$

3.3. Assay precision

3.3.1. Intra-assay

Serum samples were collected from 10 individuals known not to have consumed folic acid in the previous 24 h. A sample from each subject was then spiked with folic acid at known concentrations of 1 ng/ml, 3 ng/ml and 5 ng/ml. A further sample from each subject was left unspiked (0 ng/ml). The samples were assayed for unmetabolised folic acid as previously described. See Table 2 for results. The

Table 2
Serum folic acid assay performance

Serum	Intra-assay (n=10)	Inter-assay
Endogenous	0.00	0.00
+1 ng/ml	1.026±0.08 (7.8)	0.998±0.09 (9.02) (n=8)
+3 ng/ml	2.993±0.09 (3.03)	2.994±0.10 (3.34) (n=9)
+5 ng/ml	5.031±0.12 (2.42)	5.018±0.15 (2.99) (n=8)

Mean, SD, (C.V.%).

recovery of folic acid from the intra-assay results was as follows: 1 ng/ml 99.4%, 3 ng/ml 99.3%, 5 ng/ml 103.1%.

3.3.2. Inter-assay

A negative control along with one of the positive controls above (1 ng/ml, 3 ng/ml or 5 ng/ml) were included in 25 subsequent routine assays performed on different days (inter-assay variation, see Table 2). The recovery of folic acid from the inter-assay results was as follows: 1 ng/ml 109.5%, 3 ng/ml 78%, 5 ng/ml 102.1%.

3.4. Comparison between old and new method

In response to oral doses of folic acid (200 μg), concentrations as low as 0.32 (ng/ml) were detected by the current assay (ANOVA $P \leq 0.0001$) (see Fig. 1). No such response of serum folic acid was found using the original method.

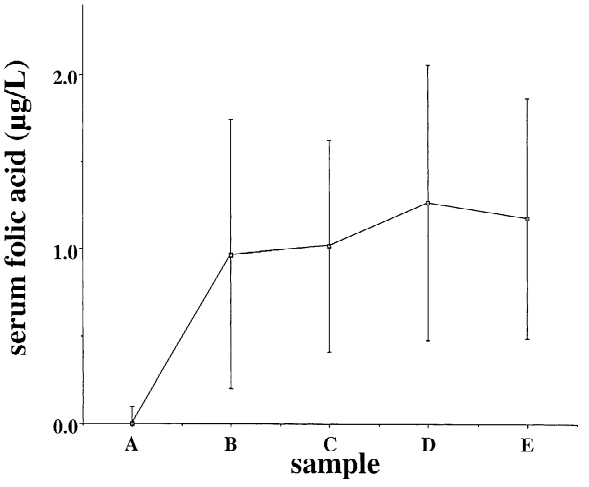

Fig. 1. Response of serum folic acid to sequential doses of folic acid in bread measured by the original and current assay. See text for details. Blood sample A refers to the preprandial blood sample. Sample B refers to the blood sample collected 1 h after the first slice of bread fortified with 200 μg of folic acid. Sample C refers to the sample collected 2 h after the first slice of bread fortified with 200 μg of folic acid. Sample D refers to the sample collected 1 h after the second slice of bread fortified with 200 μg of folic acid. Sample E refers to the sample collected 2 h after the second slice of bread fortified with 200 μg of folic acid. No such responses were detected using the previous method of serum folic acid determination.

4. Discussion

This paper describes an inexpensive, simple method with adequate sensitivity for assaying serum folic acid. This method proved to be more sensitive than previous ones [6]. For this combined HPLC/microbiological assay this was achieved by including a concentration step following HPLC chromatography. The HPLC extract was vacuum-dried and the solute was re-suspended in the minimum amount of buffer necessary for microbiological assay and recovery measurement. The introduction of this concentration step yielded greater than a three-fold improvement in the lower limit of quantitation of the assay.

The utility of the new assay compared to a previous method is illustrated by the response of unmetabolised folic acid in the serum to physiological doses of folic acid, as shown in Fig. 1. With the amounts of folic acid administered, i.e. 200 μg twice with a 4-h interval, no response was detected by the previous methodology whereas a significant increase was detected by the current method. The lack of linear response to folic acid dosage over this time period, as shown in Fig. 1, is explained by the kinetics of folic acid absorption whereby the time of maximum response (T_{max}) is 80 min [6]. Thus, the first bolus of folic acid is likely to have been eliminated from the circulation by the time the second slice was consumed.

The current methodology detected unmetabolised folic acid in serum after oral doses of 200 μg. Given this improvement in the lower limit of quantitation it is likely that the effects of smaller oral doses would be detected, even doses as low as currently adopted by the FDA fortification programme. A sensitive method such as we describe here allows the possibility of studies comparing the bioavailablity and kinetics of response of folic acid at current fortification levels in different foodstuffs.

References

[1] MRC Vitamin Research Group, Lancet 338 (1991) 131.
[2] M.A. Honein, L.J. Paulozzi, T.J. Mathews, J.D. Erickson, L.Y. Wong, J. Am. Med. Assoc. 285 (2001) 2981.
[3] P. Kelly, J. McPartlin, M. Goggins, D.G. Weir, J.M. Scott, Am. J. Clin. Nutr. 65 (1997) 1.

[4] C.E. Butterworth Jr, T. Tamura, Am. J. Clin. Nutr. 50 (1998) 353.
[5] S.D. Garbis, A. Melse-Boonstra, C.E. West, R.B. van Breemen, Anal. Chem. 73 (2001) 5358.
[6] P. Kelly, J. McPartlin, J.M. Scott, Anal. Biochem. 238 (1996) 179.
[7] A. Molloy, J.M. Scott, Methods Enzymol. 281 (1997) 43.