Methods for folate measurement: Current status, caveats and future development

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Abstract

Efforts have been made to establish reliable markers of folate status, including assays for serum folate. Development of accurate and precise folate assays has been difficult, mainly because of folate instability. Large inter-assay and inter-laboratory differences have been reported. Each laboratory has to establish its own reference interval, and comparison of results from different laboratories using different methods is difficult. Therefore, there is a need to develop novel methods for serum folate determination which are not influenced by folate degradation. We suggest such a method, which determines folate equivalents as para-aminobenzoylglutamic acid.

Introduction

Folate supplementation decreases the occurrence and recurrence rate of neural tube defects [1, 2]. This observation led to mandatory folic acid fortification of food in US and some other countries, which probably explains a substantial reduction in incident cases in these countries [3–5]. Folate deficiency in its classical form is diagnosed as megaloblastic anemia [6], but impaired folate status is also associated with chronic diseases like cardiovascular disease [7], different forms of cancer, including colorectal cancer and breast cancer [8], and cognitive impairment [9]. Because of the relationship between folate status and risk of several chronic diseases with major impact on public health, much developmental work has been done to establish and validate methods for the assessment of folate status, in particular measurement of folate concentrations in serum or plasma. In this article, we will summarize main features of such assays, their shortcomings, and suggest strategies for further developmental work in this area.

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Chemistry

All folates have a similar chemical structure, consisting of an aromatic pteridine moiety coupled via a C-9–N-10 bound to p-aminobenzoic acid (pABA), to which glutamic acid is coupled via an amide bond (Figure 1). Folate species differ with respect to the oxidation state of the pteridine ring. One-carbon substituents, including methyl, formyl or formimino groups, exist at either positions N-5 or N-10, or a methenyl or methylene bridge can connect these positions. In addition, most intracellular folates exist as polyglutamates with 5–7 glutamate residues. In serum/plasma, 5-methyltetrahydrofolate monoglutamate is the prevailing species [10, 11]. Formyltetrahydrofolate [10, 11] and folic acid are also present in serum [11]; the latter only after ingestion of supplements or fortified food containing folic acid [11, 12].

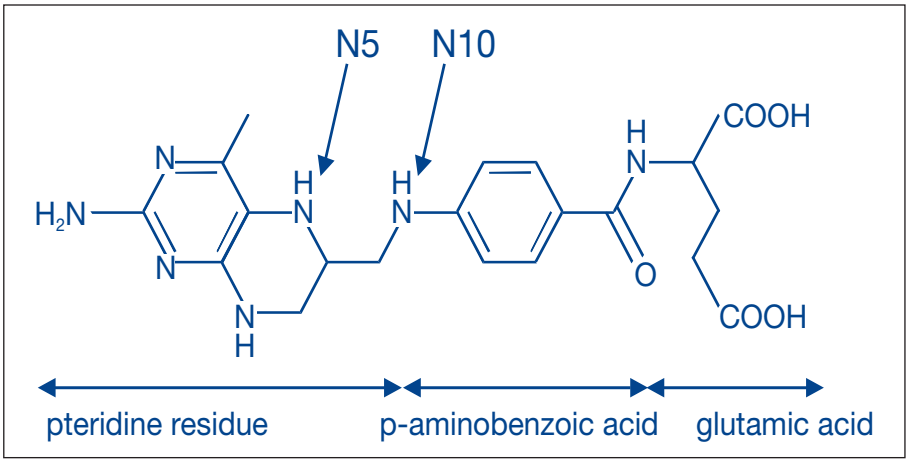

Figure 1: Chemical structure of folate.

Different methods

Microbiological assays

Most microbiological folate assays are based on the folate-dependent growth response of *Lactobacillus casei*. These assays measure total folate because *Lactobacillus casei* has a similar growth response to various folate forms [13]. Other microbiological assays detect specific folate forms and not total folate. Such assays are based on the growth response of bacteria that require particular folate species for growth [10]. When erythrocyte folate

is measured, folate polyglutamates must be deconjugated, because the growth response decreases for folate with four or more glutamate residues [14].

Protein binding assays

Assays based on folate binding protein are commonly used in clinical routine laboratories. Different assay formats have been marketed, including radioassay, chemiluminescence assay and ion capture assay. Protein binding assays can be competitive or noncompetitive. Competitive assays are based on competition between folate in the sample or standard, and a known amount of labeled folate, for the limited binding sites on a folate binding protein. In noncompetitive assays, folate in the sample is incubated with folate binding protein, and the remaining sites on the protein are quantified by titration with labeled folate. Because the binding proteins used for such assays have different affinities according to the folate polyglutamate chain length, the measurement of erythrocyte folate requires pretreatment of the sample with deconjugase [15].

Chromatographic assays

Folate, both in plasma and in erythrocytes, has been determined with HPLC using either ultraviolet [16], electrochemical [17] or fluorescence detection [18]. With LC-MS/MS, endogenous levels of 5-methyltetrahydrofolate could be measured in plasma [19]. A more recent method detects formyltetrahydrofolate and folic acid as well [11]. The latter LC-MS/MS assay has also been used for measurement of erythrocyte folate after deconjugation [20]. Recently, a LC-MS/MS method that quantifies folate together with homocysteine in serum and plasma, has been developed [21]. This may become a useful assay because of the biochemical connection between folate and homocysteine. A method for total erythrocyte folate has been published, which is based on strong acid hydrolysis of folates [22]. Folates are quantitatively converted to a common product, pABA, which then is derivatized and measured with GC-MS. The same principle has been used to measure folate as pABA with LC-MS/MS [23].

Comparison of methods

The diversity of folate species, and the instability of some, cause difficulties in folate measurement. In 1993, the commonly used folate/B12 assay of Bio Rad, Quantaphase®, turned out to be inaccurate [24]. A round robin in 1996 showed large inter-laboratory and intra-assay differences in folate measurement, especially for erythrocyte folate, but also for serum folate [25]. Later reports have confirmed that this is still a problem. A comparison of methods in 2002 showed that the mean serum folate concentration measured with the ion capture method was 20% higher then that obtained with the chemiluminescence

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assay [26]. In 2003, five automated serum and whole blood folate assays were evaluated [27]. For serum folate assays, all but one had a coefficient of variation of less then 10%, but comparison of methods showed substantial calibration differences for some of the assays. The between assay agreement was weaker for erythrocyte folate than for serum folate. Recently, four different erythrocyte folate assays, i.e. microbiological, GC-MS, radioassay and chemiluminescence assay, were compared [28]. The mean folate level obtained with the radioassay was 44–50% higher than the mean value determined with the microbiological assay.

The microbiological assay and the radioassay seem to give different concentrations of whole blood folate according to the common [29] MTHFR 677C->T polymorphism. The radioassay measures higher folate concentrations in subjects with TT compared to CC genotype, while the opposite is found when folate is measured with the microbiological assay [30]. A possible reason is that subjects with the TT genotype have a larger proportion of the total cellular folate as formylated forms of folate [31]. Such folate species may be overestimated by the folate binding protein assay, because they have higher affinity than 5-methyltetrahydrofolate for the binder [32] .

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Evaluation of methods

The microbiological assay based on *Lactobacillus casei* has been considered the gold standard because *Lactobacillus casei* has similar growth response to all the different forms of folate monoglutamate. However, the assay is time-consuming and difficult to perform. Therefore, it is not commonly used in clinical laboratories, even though the assay has been improved and automated [33]. Antibiotics may also interfere with the result. Automated protein binding assays are easy to use, but there has been serious concern about the differences in affinity for the different folates to the binding proteins [15]. A disadvantage of microbiological and protein binding assays is that they can only measure total folate. With HPLC and LC-MS/MS, the different folate forms are separated and detected. With LC-MS/MS, isotopically labeled internal standards should be used; however, including internal and external standards for all the folates can be impractical because of the instability of some folate species. Finally, the equipment is expensive.

Measuring erythrocyte folate as pABA with GS-MS [22] or LC-MS/MS [23] could overcome some of the problems with folate instability, as long as the pABA moiety of the folate is intact. It is a prerequisite that the different folate forms are totally recovered as pABA, and that other pABA precursors than folate do not exist. With erythrocyte folate assays based on measurement of pABA, no conjugase treatment is required, which could be a major advantage considering variable effectiveness of deconjugation [34, 35].

Plasma/serum or erythrocyte folate

It has been discussed whether plasma/serum or erythrocyte folate is the optimal marker for folate status [36–38]. Plasma/serum folate is influenced by recent dietary intake, while erythrocyte folate is considered to reflect the overall and long-term folate status [39]. Technically, it is more convenient to measure plasma/serum folate, and some serum folate assays (but not erythrocyte folate assays) allow the simultaneous determination of serum cobalamin. The erythrocyte folate assay is complex and requires hemolysate preparation that is difficult to control. The various folate species must be deconjugated to monoglutamate forms prior to detection. This reaction is catalyzed by endogenous deconjugase, is pH sensitive and difficult to standardize [34]. Folate might also be trapped irreversibly by binding to hemoglobin [35]. The calibrators used (usually folic acid in aqueous matrix) do not undergo the various steps in the assay, and do not adjust for precipitation, hemolysis, and binding of folates to hemoglobin.

Developmental work

Traditional folate assays do not measure all folate forms present in serum samples collected and stored under conditions causing degradation or oxidation of labile folates, including 5-methyltetrahydrofolate, the prevailing folate species in serum. To be able to measure partly degraded and modified serum folates as well, we investigated the possibility to determine folate as pABA equivalents after strong acid hydrolysis. This was not successful because serum from some individuals contains pABA precursor(s) that were not biologically active folate. We therefore developed a serum folate assay, which is based on quantitative conversion of folate into para-aminobenzoylglutamic acid (pABG), after oxidation followed by limited acid hydrolysis. pABG is quantitated by LC-MS/MS. With this assay we have measured folate concentrations in serum samples stored for 30 years at –20 °C.

Conclusion

Despite development of several new folate assays and improvement of existing ones, there is a need for better standardization of folate testing. In the future, to be able to compare inter-assay and inter-laboratory results, common standard reference materials and calibrators will be important. Since there is a general difficulty in standardizing immuno- and bioreceptor-based assays, chromatographic and chemical methods including the most important folate species in fresh and stored biological samples should be developed. Such methods should serve as reference techniques, and the pABG folate assay is a candidate method.

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