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Suitability of methylmalonic acid and total homocysteine analysis in dried bloodspots

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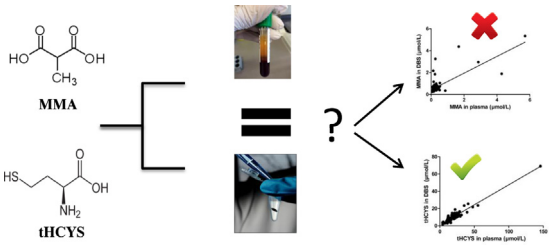
HIGHLIGHTS

- DBS assay offer sufficient sensitivity to measure MMA and tHCYS concentrations.
- DBS matrix is superior for tHCYS stability.
- Paired plasma and DBS samples were compared.
- DBS samples for measurement of tHCYS is a good alternative approach for plasma.
- For MMA, no correlation between DBS and plasma exist in the lower ranges.

GRAPHICAL ABSTRACT



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ABSTRACT

Methylmalonic acid (MMA) and total homocysteine (tHCYS) concentrations are used to detect acquired and inborn errors of cobalamin (vitamin B12, Cbl) metabolism and to evaluate the effect of therapeutic interventions. Dried blood spot sampling offers a patient-friendly and easy alternative to plasma sampling. However, dried blood spot concentrations are not necessarily equal to plasma concentrations. Therefore, the objective of this work was to establish the relationship between MMA and tHYS dried blood spot and plasma concentrations to facilitate clinical implementation of dried blood spot sampling. MMA and tHCYS in both plasma and DBS were validated on ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). While position of the punch (in DBS) did affect tHCYS concentration, no influence of hematocrit (Ht) and blood volume on both MMA and tHCYS concentrations was observed. The plasma assay performed better than the DBS assay by most criteria. However, the DBS matrix was superior for tHCYS stability. Paired plasma and DBS samples were obtained from patients suspected for Cbl deficiency and from patients with a known inborn error of metabolism affecting MMA or tHCYS concentration. Based on the strong correlation of tHCYS in both matrices ($y = 0.46 \pm 1.12$ ($r^2 = 0.91$)), determination of tHCYS in plasma can be replaced by tHCYS in DBS. However, for MMA, a

Abbreviations: MMA, methylmalonic acid; Cbl, cobalamin; tHCYS, total homocysteine; DBS, dried blood spots.

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correlation in the higher (pathological) range of MMA exist, but no correlation was observed in the lower ranges. Therefore the added value of MMA concentrations in DBS is currently unknown and should be further investigated.

1. Introduction

Cobalamin (vitamin B12, Cbl) functions as a methyl donor, works with folic acid in the synthesis of DNA and red blood cells and is vitally important in maintaining the myelin sheath that surrounds nerve cells. Cbl serves as cofactor for two important cellular enzymatic reactions. In the first reaction, adenosyl–Cbl catalyses the formation of succinyl–CoA from methylmalonyl–CoA. The second reaction concerns remethylation of homocysteine to methionine by methyl–Cbl. Both acquired and congenital disturbances of Cbl metabolism exist. Inborn errors of Cbl metabolism included those affecting its absorption, (intrinsic factor deficiency, Imerslund-Gräsbeck syndrome), its transport (*trans*-Cbl deficiency) or the intracellular metabolism affecting either adenosyl–Cbl synthesis (cblA and cblB), methionine synthase function (cblE and cblG) or both (cblC, cblD and cblF) [1]. Recently, two new inborn errors of metabolism of Cbl metabolism have been described [2,3].

Acquired Cbl deficiency may be caused by inadequate intake, abnormal absorption, or increased requirements [4]. There are a number of drugs that interfere with Cbl absorption and deplete Cbl stores. For example, the antidiabetic agent metformin [5]. The exact prevalence of Cbl deficiency is unknown but is estimated to be approximately 20% of the general population [6], is more common among elderly people [7] and probably under-diagnosed. Cbl deficiency can cause a wide spectrum of clinical features including hematologic, neurologic, psychiatric, gastrointestinal, dermatologic and cardiovascular manifestations. It can be treated by oral and/or intramuscular vitamin B12 formulations [8,9]. Early diagnosis and prompt treatment can often reverse clinical symptoms and laboratory abnormalities disappear.

Screening for Cbl deficiency is hampered by the poor sensitivity of the existing total vitamin B12 assay [10]. Both MMA and tHCYS are markers used for the diagnosis and follow-up of Cbl deficiency [11]. In fact, it has been reported that MMA and tHCYS levels preceded a decrease in plasma vitamin B12 concentration and can therefore act as an early marker for tissue Cbl deficiency [12]. Elevated tHCYS and MMA indicate Cbl deficiency with a sensitivity and specificity of 94% and 99%, respectively [6]. The overall diagnostic utility of tHCYS and MMA has been reviewed in detail [13].

Plasma is probably the most widely used matrix to determine metabolite concentrations. Over the last years, dried blood spots (DBS) have become a popular alternative. The interest in using DBS for clinical studies has increased and has been applied in pharmacokinetic studies, toxicokinetic studies, epidemiological investigations and is used for therapeutic drug monitoring. The increased use and acceptation of DBS sampling have been driven by the simplicity of the technique for collection, storage and transport of the samples [14]. An important benefit of DBS is the small amount of material needed. This is not only in favor of sick and small infants but also is necessary when repeating measurements (follow up treatment).

To date, several methods have been described for determination of tHCYS in DBS using high performance liquid chromatography (HPLC) with fluorescence detection [15,16] and electrochemical coulometric array detection [17]. In addition, MMA in DBS can be analyzed by GC/MS [18] and HPLC with intramolecular-excimer fluorescence derivatization [19]. HPLC–MS/MS is now considered the method of choice for the quantitative determination of several metabolites and has been successfully applied for both tHCYS [20,21] and MMA in DBS [21,22]. To improve efficiency, we combined both metabolites in one assay and analyzed them with UPLC MS/MS. The UPLC procedure provided improved chromatographic parameters resulting in significantly increased sample throughput including lower solvent consumption and lower limits of detection (LODs) for most of target analytes compared to common method employing HPLC separation. Although MMA and tHCYS measurements in DBS have been reported, several factors that potentially affect DBS assay quantitation (punch location, impact of hematocrit (Ht) and volume of the blood sample) were not investigated. The objective of this work was to compare MMA and tHCYS concentrations between paired plasma and DBS samples, since metabolites in DBS and plasma do not always correlate [23].

2. Material and methods

2.1. Reagents

MMA was purchased from Fluka chemika (Buchs, Switzerland) and isotopically labeled MMA (MMA-D_3) was purchased from Isotec, Sigma (Steinheim, Germany). DL homocysteine, dithiothreitol (DTT) and acetyl chloride were purchased from Sigma (Steinheim, Germany) and isotopically labeled homocystine-D_8 was purchased from Buchem (Apeldoorn, the Netherlands). Formic acid and *n*-butanol were purchased from Merck (Darmstadt, Germany) and acetonitrile was obtained from Biosolve (Valkenswaard, the Netherlands). The filter paper used for the sample collection was grade 903 (Whatman no. 903 Protein SaverTM cards, formerly Schleicher & Schuell, Keene, USA).

2.2. Subjects

Heparin containing blood samples were obtained from patients admitted for analysis of MMA and/or tHCYS. As part of the validation, blood samples were collected from a patient with methylmalonic acidemia and from a patient with methylenetetrahydrofolate reductase (MTHFR) deficiency (both confirmed by mutation analysis) who had persistently increased concentrations of MMA or tHCYS respectively.

2.3. Sampling

Blood samples were collected by venous puncture into heparin containing tubes (Li-heparine tube, Greiner Bio-One the Netherlands). Blood was rotated during 30 min. The homogenous blood is allowed to drop from a pipette onto the center of the guide circle on the DBS card. The pipette should not touch the card but remain a few millimeters above the card to allow equal distribution of the blood drop across the card. A sufficient quantity of blood was used to soak through and completely fill a pre-printed circle.

The blood tubes were then centrifuged and plasma was stored at −80 °C until further analysis. The spotted Guthrie card filter papers were left to dry at least 4 h at room temperature and stored at −20 °C in a foil bag with a desiccant package pending further analysis.