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Original Article

Simultaneous quantitation of folic acid and 5-methyltetrahydrofolic acid in human plasma by HPLC–MS/MS and its application to a pharmacokinetic study ☆

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ABSTRACT

A sensitive method based on high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed for the simultaneous determination of folic acid (FA) and its active metabolite, 5-methyltetrahydrofolic acid (5-M-THF), in human plasma. The analytes were extracted from plasma with methanol solution containing 10 mg/mL of 2-mercaptoethanol and 0.025% (v/v) ammonium hydroxide. FA and 5-M-THF were more stable after the addition of 2-mercaptoethanol and ammonium hydroxide in the sample preparation procedures of this study than they were in the previously published methods. Chromatographic separation was performed on a Hedera ODS-2 column using a gradient elution system of acetonitrile and 1 mM ammonium acetate buffer solution containing 0.6% formic acid as mobile phase. LC–MS/MS was carried out with an ESI ion-source and operated in the multiple reaction monitoring (MRM) mode. The assay was linear over the concentration ranges of 0.249–19.9 ng/mL for FA, and 5.05–50.5 ng/mL for 5-M-THF. The developed LC–MS/MS method offers increased sensitivity for quantification of FA and 5-M-THF in human plasma and was applicable to a pharmacokinetic study of FA and 5-M-THF.

1. Introduction

Folates belong to B vitamins and are essential elements in the diet. They are a group of compounds derived from food-stuffs. The key folate forms include folic acid (FA), 5-methyltetrahydrofolic acid (5-M-THF), 5-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate, S-adenosylmethionine, and S-adenosylhomocysteine [1]. Previous studies showed that folate deficiency was associated with increased risk of neural tube defects [2,3], coronary heart disease [4,5], certain types of cancer [6,7], Down's syndrome [8], and red-cell aplasia [9]. Nowadays FA is used as an oral supplement by patients with these disorders and recommended to women of childbearing age to reduce the risk of neural tube defects. FA is absorbed in the small intestine and is reduced to the metabolically active tetrahydrofolate forms inside the cells. 5-M-THF is the most predominant active metabolite, which accounts for approximately 98% of folates in human plasma [10].

The structures of FA and 5-M-THF are shown in Fig. 1. The main challenge in FA and 5-M-THF quantification is their instability due to the cleavage of the C^9–N^{10} covalent bond and the reduction of the pterin ring [11], because they are easily degraded under different conditions of temperature [12], pH [12,13], oxygen [14] or light [15]. Just as early research reports, FA and 5-M-THF are more stable in alkaline conditions than in acidic conditions [16].

Folates in human serum and plasma have been measured by various chromatographic methods, including high performance liquid chromatography (HPLC) [17], liquid chromatography–mass spectrometry (LC–MS) [18], and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [1,19–24]. However, these methods were not all convenient for routine analysis due to various reasons such as employing large sample volumes [19], having long retention time [21] or applying time-consuming extraction procedures (purified by folate binding protein affinity columns[19] or solid-phase extraction (SPE) [21–24]). Moreover, these reports did not put emphasis on the stability of FA and 5-M-THF during the

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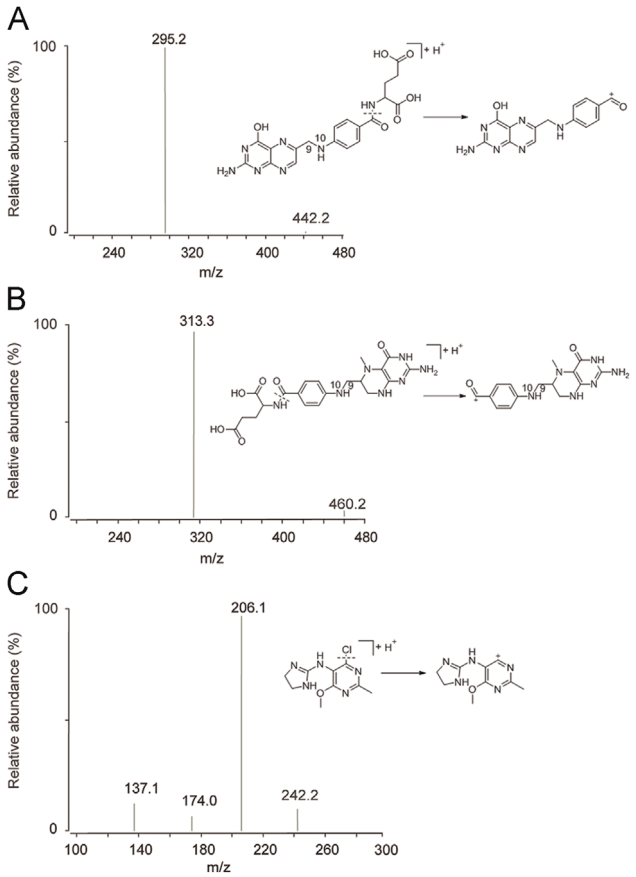

Fig. 1. Chemical structures of (A) FA, (B) 5-M-THF and (C) IS and their proposed fragmentation patterns.

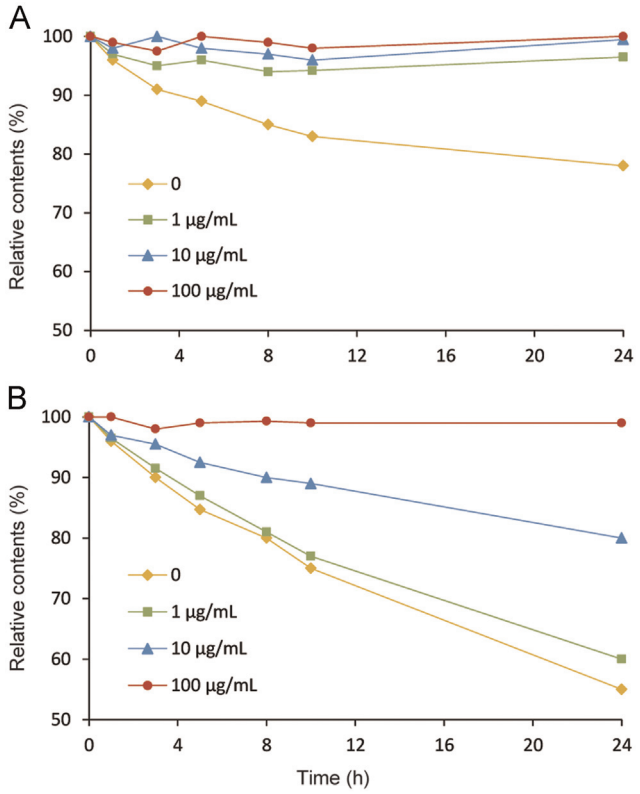

Fig. 2. The degradation of (A) FA (9.95 ng/mL) and (B) 5-M-THF (20.2 ng/mL) in plasma at different concentration levels of 2-mercaptoethanol at room temperature.

sample preparation process. This paper describes a reliable and reproducible LC–MS/MS method for the simultaneous quantification of FA and 5-M-THF in human plasma and it has been applied to the pharmacokinetic study of FA and 5-M-THF in healthy Chinese male volunteers.

2. Materials and methods

2.1. Chemicals and reagents

The reference standard of FA (89.7%) was purchased from the National Institute for Food and Drug Control (Beijing, China), reference standard of moxonidine (99.2%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and reference standard of 5-M-THF (98.1%) was supplied by Toronto Research Chemical Inc. (Ontario, Canada). Acetonitrile and methanol were of gradient grade for liquid chromatography (Merck, Germany). Formic acid, ammonium hydroxide and ammonium acetate were of analytical grade purity and were purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). 2-Mercaptoethanol was purchased from Shanghai Sigma Metals, Inc. (Shanghai, China). FA tablets containing 5 mg of FA were purchased from Jiangsu Yabang Epson Pharmaceutical Co., Ltd. (Yancheng, China) and FA tablets containing 0.4 mg of FA were purchased from Beijing Slaine Pharmaceutical Co., Ltd. (Beijing, China).

2.2. Instrumentation and chromatographic conditions

The liquid chromatography was performed on an Agilent 1200 Series liquid chromatography (Agilent Technologies, Palo Alto, CA, USA), which comprised an Agilent 1200 binary pump (model G1312B), a vacuum degasser (model G1322A), an Agilent 1200 autosampler (model G1367C), and a temperature controlled column compartment (model G1330B). The chromatographic separation was carried out on a Hedera ODS-2 analytical column (150 mm × 2.1 mm i.d, 5 μm; Hanbon Science and Technology, Huaian, Jiangsu, China) with a security guard C_{18} column (4 mm × 2.0 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA) at the temperature of 38 °C.

The mobile phase consisted of acetonitrile (solvent A) and 1 mM ammonium acetate buffer solution containing 0.6% formic acid (solvent B) was delivered at 0.40 mL/min according to the following programs: 0–0.8 min (89% B), 1.0–2.0 min (30% B), 2.2–5.0 min (10% B), and 5.2–10.0 min (89% B). The column effluent was directed into the mass spectrometer at the time interval of 0–4.0 min, otherwise to waste. Autosampler temperature was maintained at 4 °C and an injection volume of 8 μL was used in each run.

The LC system was coupled with an Agilent 6410B triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) equipped with an electrospray source (model G1956B). The electrospray ionization source was set with a drying nitrogen gas flow of 12 L/min, nebulizer pressure of 40 psig, drying gas temperature of 350 °C, capillary voltage of 4.0 kV in positive ion mode. The fragmentor voltages for FA, 5-M-THF and IS were 90, 105 and 100 V, respectively. The fragmentation transitions for the multiple reaction monitoring (MRM) were m/z 442.2→295.2 for FA with a collision energy (CE) of 22 eV, m/z 460.2→313.3 for 5-M-THF with a CE of 20 eV, and m/z 242.1→206.1 for IS with a CE of