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Original article

Separation, determination of six impurities in methotrexate drug substance using ultra-performance liquid chromatography

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ABSTRACT

Methotrexate (MTX) is an antineoplastic therapeutic medicine as antimetabolite of folic acid. In this paper, a sensitive and rapid ultra-performance liquid chromatographic (UPLC) method was developed and validated for the separation and determination of impurities in MTX drug substances. The UPLC method was accomplished on an Agilent Zorbax Extend C-18 (50 mm × 4.6 mm, 1.8 μm) with a gradient elution system composed of sodium dihydrogen phosphate in water (20 mmol/L, pH 3.0) and acetonitrile. The flow rate was 2.2 mL/min. The method was validated. The calibration curves displayed good linearity ($r > 0.999$) within the tested concentration ranges. The limit of detection (LOD) and limit of quantification (LOQ) of the six analytes were all less than 0.774 μg/mL and 1.03 μg/mL. The relative standard deviation (RSD) for intra- and inter-day precision of the six analytes was less than 9.8%, including at the LOQ. The average recovery ranged from 95.2% to 103% except at the LOQ, where recovery ranged from 82.7% to 117%. The validated method was successfully used to determine the relative abundance of six impurities in the MTX drug substances.

1. Introduction

Methotrexate ((2S)-2-[[4-[[(2,4-diaminopteridin-6-yl)methyl]-methylamino]benzoyl]amino]pentanedioic acid; MTX) is an anti-neoplastic therapeutic medicine that acts as an anti metabolite of folic acid [1–3]. It is mainly used in the treatment of meningeal leukemia, psoriasis, and rheumatoid arthritis. The most common adverse effects of MTX include ulcerative stomatitis, low white blood cell count, nausea, abdominal pain, fatigue, fever, dizziness, acute pneumonitis, and rarely, pulmonary fibrosis [3,4].

At present, the impurity limit for MTX has definite provisions in the pharmacopoeia of each country [1,5–7]. For example, in the United States Pharmacopeia and Chinese Pharmacopeia, the total amounts of all impurities should not exceed 2.0% while each single impurity should not exceed 0.5%. In the European Pharmacopoeia (EP) and British Pharmacopoeia (BP), twelve impurities are specified (impurities A–L). The EP and BP both set specific limits for each impurity. In detail, impurity C is less than 0.5%, impurities B and E are less than 0.3%, impurities H and I are less than 0.2%, and the other impurities (impurities A, D, F,G, J, K, L) are less than 0.05%. In addition, the sum of impurities, other than B, C, and E, is less than 0.5%. In our previous study of impurities in MTX [8], we found that the impurity profile of MTX drug substances sold in China differed from the reported profiles. We also detected a new impurity, methotrexate 5-ethyl ester, which is not included in the EP or BP. Surprisingly, this impurity was the most abundant impurity in some MTX drug substances. Herein, it is necessary to develop a method to determine impurities in MTX drug substances for improving the quality control standards.

To date, several methods have been described for testing MTX in bulk material, in pharmaceutical dosage forms and in biological samples. These methods included, high-performance liquid chromatography (HPLC) [1,5–7,9–12], capillary electrophoresis [13], high-performance liquid chromatography tandem mass spectrometry (HPLC–MS) methods [8,14,15]. However, very few methods have been described in the context of testing impurities in MTX [1,2,5–9], and the run-time of existing method is commonly very long. Due to ultra-performance liquid chromatography (UPLC) with high separation resolution and rapid analysis characterization, a simple and rapid UPLC method was developed to simultaneously determine the six main impurities of MTX drug substances, namely *N*-methylfolic acid (impurity C in

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EP and BP), 4-amino-N_{10}-methylpteroic acid (impurity E in EP and BP), N_{10}-methylpteroic acid (impurity D in EP and BP), methotrexate 5-methylester (impurity H in EP and BP), methotrexate dimethyl ester (impurity J in EP and BP) and methotrexate 5-ethyl ester (termed impurity N, new impurity). To our knowledge, this is the first report describing the use of UPLC to detect impurities in MTX drug substances. The method was validated in accordance with the International Conference on Harmonisation of Technical Registration of Pharmaceuticals for Human Use (ICH) Guidelines [16], and was successfully used to detect MTX impurities in drug substances.

2. Experimental

2.1. Instruments and materials

The assay was performed on a LC-20A system (Shimadzu, Kyoto, Japan), comprising a LC-20AD XR pump, a SIL-20A XR autosampler, a CTO-20A column oven, and a SPD-M20A UV detector. LC solution software was used for LC control and data acquisition (Kyoto, Japan).

The structures of the six impurities are shown in Fig. 1. Five batches of MTX drug substances (batches 09002, 09006, 040601, 040101, and 061005) were obtained from four different companies. Batches 09002 and 09006 were obtained from market sold in China. The other three batches were provided by the National Institutes for Food and Drug Control and were collected from two different companies (batch 040601 and 040101 were from same company). The MTX reference standard was obtained from the National Institutes for Food and Drug Control (purity: 99.4%). The MTX impurities reference standards were synthesized by Professor Song Wu using organic chemistry methods and their structures were confirmed based on their MS, ^{1}H NMR and ^{13}C NMR data. The MTX impurities reference standards were separated and purified in our lab. The purity of these compounds was more than 95% (determined by HPLC).

Acetonitrile of LC/MS reagent grade was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Deionized water was purified using a Millipore water purification system (Millipore, Billerica, MA, USA). Analytical grade acetic acid was obtained from Merck Inc. (Darmstadt, Germany). Sodium dihydrogen phosphate and phosphoric acid of analytical grade were purchased from Beijing Chemical Corp. (Beijing, China). Analytical grade dimethylsulfoxide (DMSO) was obtained from Acros Organics Corp. (Geel, Belgium)

2.2. UPLC analytical conditions

Chromatographic separation was performed using an Agilent Zorbax Extend-C18 column (4.6 mm × 50 mm, 1.8 μm). The mobile phase consisted of solvent A (deionized water with 20 mmol/L sodium dihydrogen phosphate, adjusted to pH 3.0 with phosphoric acid) and solvent B (acetonitrile), delivered at a flow rate of 2.2 mL/min. Gradient elution was started at 8% B, which was held until 4.0 min, followed by an increase to 12% B at 9 min, and maintained until 11 min. The post-run time is 5 min. The column temperature was maintained at 40 °C and the sample injection volume was 3 μL. Detection was carried out at 305 nm.

2.3. Preparation of system suitability solution

Standard stock solutions of the six impurities were prepared in DMSO at a concentration of 500 μg/mL, respectively. Appropriate amounts of the stock solutions were then mixed and diluted with DMSO to prepare working solutions with a desired concentration about 25 μg/mL. Additionally, 25 mg of the MTX reference standard were accurately weighed, transferred to a 5 mL volumetric flask, dissolved and diluted to the volume with the working solution (25 μg/mL) of six impurities.

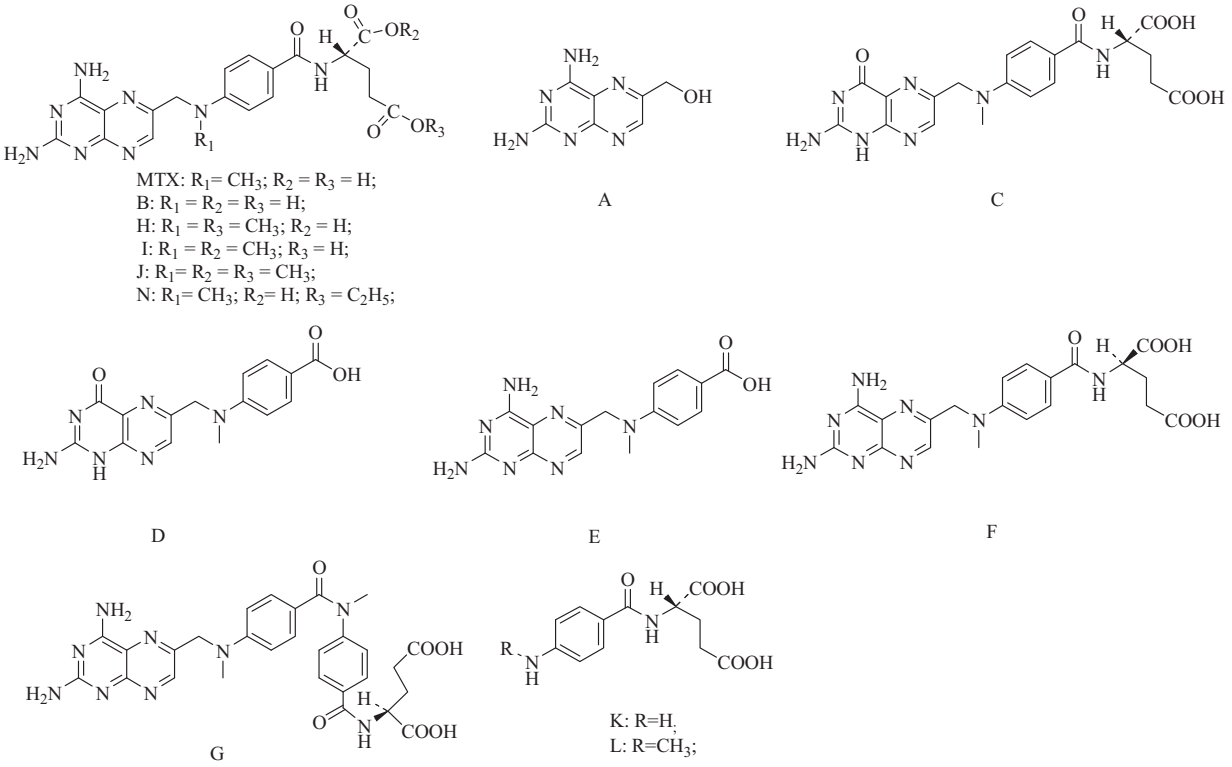

Fig. 1. Chemical structures of 13 impurities and MTX. The letters assigned to A–L are identical to EP and BP, while impurity N is a new impurity.