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A new quantification method for assessing plasma concentrations of pemetrexed and its polyglutamate metabolites

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ABSTRACT

Currently no quantification method exists for potentially therapeutically relevant polyglutamate metabolites of the drug pemetrexed which is used for the treatment of lung carcinoma patients.

We developed and tested an LC–MS/MS-based analytical assay that uses isotope-labeled internal standards to quantify pemetrexed and its (poly)glutamate metabolites in clinical human plasma samples of lung carcinoma patients.

UHPLC chromatography and triple quadrupole mass spectrometry showed an LLOQ of 0.2 nmol/L for pemetrexed and an LLOQ of 0.5 nmol/L for the two metabolites (one glutamate and two glutamate moieties covalently bound to the pemetrexed molecule, for which no other quantification methods have previously been published). The recoveries for PMTX and its metabolites ranged between 30% and 67%. Precision and accuracy at a concentration of 20 nmol/L for all four analytes was well below 15% CV. The precision (RSD) in the biological replicates of the separate days (within-run precision) as well as the reproducibility over several days (between-run precision), tested in the range of 5–250 nmol/L, were all below 15%. Autosampler, benchtop and freeze-thaw cycle stability of the analytes was also demonstrated. To illustrate the new assay in a relevant biological context, concentrations of pemetrexed and the two metabolites were quantified in plasma samples of lung carcinoma patients treated with pemetrexed.

The assay is straightforward, relatively easy to perform, and has potential for use in therapeutic drug monitoring in non-small cell lung carcinoma patients.

1. Introduction

Pemetrexed (Alimta®; *N*-[4-[2-[2-amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-*d*]pyrimidine-5-yl ethyl] benzoyl-L-glutamic acid]; Fig. 1) is a multi-targeted anti-folate drug that is used in the treatment of diseases such as malignant pleural mesothelioma and non-small-cell lung cancer [1–4]. While the main intracellular targets of pemetrexed and methotrexate differ [5,6], they are both antifolate drugs which exert antineoplastic effects by disturbing the folate-dependent metabolic processes necessary for cellular replication. Although the mechanisms of action of both drugs are similar, pemetrexed may have additional effects due to its rapid intracellular polyglutamation, i.e. the enzymatic attachment of glutamic acid residues to the pemetrexed molecule [7].

Interestingly, polyglutamated metabolites of other anti-folate drugs tend to have a higher inhibitory potential towards the main enzymatic target thymidylate synthase; their intracellular retention also tends to be enhanced [8,9]. While the role of polyglutamated metabolites of methotrexate in disease development and treatment response has been studied extensively [10–14], pemetrexed polyglutamates have been studied less, possibly because, unlike the situation with methotrexate, there is no reliable method described for measuring them.

Abbreviations: Fmoc, fluorenylmethyloxycarbonyl; FPGS, folylpoly-gamma glutamase synthase; MSNT, 1-(2- mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole; PMTX, pemetrexed; PMTXPG1, Pemetrexed-monoglutamic acid; PMTXPG2, pemetrexed-diglutamic acid; PMTXPG3, pemetrexed-triglutamic acid; RSD, relative standard deviation; TCA, trichloroacetic acid; % CV, coefficient of variation.

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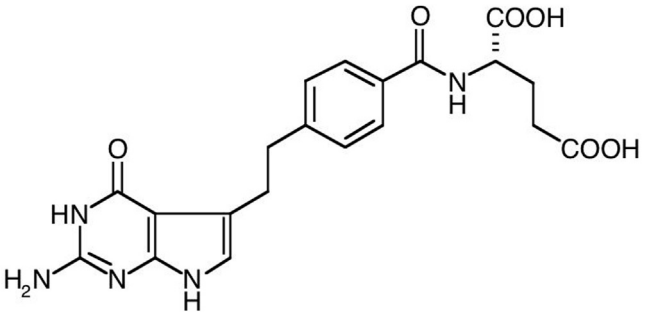

Fig. 1. The structural formula of pemetrexed.

Assessment of therapeutic plasma drug concentrations offers a useful tool for monitoring drug dosage, avoiding drug toxicity and obtaining therapeutic efficacy. Pemetrexed concentrations can be determined by two methods: high performance liquid chromatography coupled with ultraviolet readout or mass spectrometry detection, including MALDI-QqQ-MS/MS [15–18]. To our knowledge, however, no method has been reported for quantifying pemetrexed metabolites (such as pemetrexed polyglutamates) in human plasma or any other biofluid or tissue. The concentrations of these metabolites could provide important additional information regarding the treatment of patients, as pemetrexed is chemically very similar to methotrexate and for methotrexate it is the polyglutamated metabolites that are most predictive of response to treatment [10,19], it is anticipated that these assumed metabolites of pemetrexed could have therapeutic effects.

Here, we describe a new UHPLC mass spectrometry assay for quantifying pemetrexed and pemetrexed polyglutamate metabolite concentrations in human plasma of non-small-cell lung cancer patients.

2. Materials and methods

2.1. Reference materials and chemicals

The following chemicals were synthesized by Pepscan Therapeutics (Lelystad, the Netherlands) in the manner described below: *N*-[4-[2-[2-amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-*d*]pyrimidine-5-yl ethyl] benzoyl]-L-glutamic acid (Pemetrexed, PMTX), *N*-[4-[2-[2-amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-*d*]pyrimidine-5-yl ethyl] benzoyl]-L-diglutamic acid (PMTXPG1), *N*-[4-[2-[2-amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-*d*]pyrimidine-5-yl ethyl] benzoyl]-L-triglutamic acid (PMTXPG2) and *N*-[4-[2-[2-amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-*d*]pyrimidine-5-yl ethyl] benzoyl]-L-tetraglutamic acid (PMTXPG3) and corresponding heavy labeled stable isotope analogues ($^{13}C_5$, ^{15}N (6 Da mass difference)). Fluorenylmethyloxycarbonyl (Fmoc) –Glu(OtBu)-OH (13C5, 15N) was coupled to a WANG-Resin using the standard MSNT method (1-(2-mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT) in the presence of *N*-methylimidazole). The polyGlu chains were then elongated using standard Fmoc chemistry (HBTU/HOBt as coupling reagent and 20% piperidine to remove the Fmoc). This was followed by the coupling of 4-[2-(2-amino-4,7-dihydro-4-oxo-1H-pymol[2,3-D]pyrimodin-5-yl)ethyl]benzoyl. Finally, the compound was cleaved from the resin using trifluoroacetic acid/$H_2$0/1,2-ethanedithiol (95/2.5/2.5).

The compound was then purified by reverse HPLC. The PMTX and PMTXPG stock solutions were all prepared at a concentration of 0.5 mg/mL in water. High-purity water, 0.1% formic acid in water, and 0.1% formic acid in acetonitrile were supplied by Biosolve (Valkenswaard, the Netherlands). Trichloroacetic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

Samples were prepared for measurement according to the following protocol: 60 μL of EDTA plasma was added to 20 μL of water and 20 μL internal standards (all 4 heavy labeled compounds, each at 2000 nmol/L). Subsequently, 100 μL 2 M trichloroacetic acid (TCA) was added for protein precipitation. After centrifugation of the samples for 10 min at 8000 rpm (5223*g*) in a 5415C centrifuge (Eppendorf, Hamburg, Germany), 100 μL of the supernatant was pipetted into an LC vial for measurement. For the measurements of the standards, the 20 μL water was replaced with a spiked solution of the four analyte compounds at known concentrations in HPLC-grade water.

2.3. UHPLC mass spectrometry system

The liquid chromatography system was a Dionex Ultimate 3000 UHPLC system, consisting of an Ultimate 3000 RS pump, an Ultimate 3000 flow manager, and an Ultimate 3000 RS autosampler (all Thermo Scientific, Sunnyvale, CA, USA). The chromatographic separation was performed on a Kinetex 2.6 μ C18 100 Å column (Phenomenex, Torrance, CA, USA) protected by a Security Guard Cartridge System (KJ0-4282, Phenomenex, Torrance, CA, USA), which was kept at a constant temperature of 40 °C. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The measurement consisted of an 11-min gradient program starting at 10% solvent B during the injection of the sample, whilst the flow was set at 0.6 mL/min. Subsequently, the solvent B concentration was changed to 80% at 0.3 min. This percentage remained constant until 7.0 min, when the %B was changed again and set to gradually reducing to 10% at 9.1 min, where it stayed constant until the end of the gradient at 11.0 min. The injection volume was 25 μL. The system was operated using Dionex Chromeleon software version 6.80.

Mass spectrometry measurements were performed online on an API4000 (AB Sciex, Framingham, MA, USA) and were operated using the Analyst software package (version 1.6.2, AB sciex, Framingham, MA, USA) in a Selected Reaction Monitoring (SRM) experiment. The fragment ion observed was the same for all analytes and standards (*m*/*z* 281.2, Fig. 1). Collision gas (nitrogen) was set at 12, curtain gas at 35 and ion source gas at 40. The ion spray voltage was set at

Table 1
The parent mass, transition and optimized parameters for all analytes and internal standards.

Compound	Q1 *m*/*z*	Q3 *m*/*z*	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collison cell exit Potential (CxP)
PMTX	428.0	281.2	64	10	28	18
PMTXPG1	557.0	281.2	75	10	39	18
PMTXPG2	686.1	281.2	89	10	49	18
PMTXPG3	815.2	281.2	100	10	61	18
IS-PMTX	434.0	281.2	64	10	28	18
IS-PMTXPG1	563.0	281.2	75	10	39	18
IS-PMTXPG2	692.1	281.2	89	10	49	18
IS-PMTXPG3	821.2	281.2	100	10	61	18