Highly sensitive quantification of pemetrexed in human plasma using UPLC-MS/MS to support microdosing studies

Eleonora W. J. van Ewijk-Beneken Kolmer1 | Marga J. A. Teulen1 | Rene J. Boosman2 | Nikki de Rouw1,3 | Jacobus A. Burgers2 | Rob ter Heine1

1Department of Pharmacy and Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands
2Department of Pharmacy and Pharmacology, Antoni van Leeuwenhoek—The Netherlands Cancer Institute, Amsterdam, The Netherlands
3Department of Pharmacy, Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands

Correspondence
Rob ter Heine, Department of Pharmacy and Radboud Institute for Health Sciences, Radboud University Medical Center, 864, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.
Email: r.terheine@radboudumc.nl

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Abstract
Pemetrexed is an antifolate drug approved for the treatment of non-small-cell lung cancer and mesothelioma. Assessing pemetrexed pharmacokinetics after administration of a microdose (100 μg) may facilitate drug–drug interaction and dose individualization studies with cytotoxic drugs, without causing harm to patients. Therefore, a highly sensitive bioanalytical assay is required.

A reversed-phase ultra-high performance liquid chromatography method was developed to determine pemetrexed concentrations in human ethylenediaminetetraacetic acid-plasma after microdosing. [13C5]-Pemetrexed was used as the internal standard.

The sample preparation involved solid-phase extraction from plasma. Detection was performed using MS/MS in a total run time of 9.5 min.

The assay was validated over the concentration range of 0.0250–25.0 μg/L pemetrexed. The average accuracies for the assay in plasma were 96.5 and 96.5%, and the within-day and between-day precision in coefficients of variations was <8.8%. Extraction recovery was 59 ± 1 and 55 ± 5% for pemetrexed and its internal standard. Processed plasma samples were stable for 2 days in a cooled autosampler at 10°C.

The assay was successfully applied in a pharmacokinetic curve, which was obtained as a part of an ongoing clinical microdosing study.

KEYWORDS
microdosing, pemetrexed, plasma, tandem mass spectrometry, ultra-high performance liquid chromatography

1 | INTRODUCTION

Pemetrexed (Figure 1) is a multtargeted antifolate drug mainly used to treat lung cancer, either as monotherapy or in combination with other chemotherapy or immunotherapy (Planchard et al., 2018). Pemetrexed is a classic cytotoxic agent, where balancing the dual risk of subtherapeutic and toxicity is often a challenge. With drugs like pemetrexed, dosing is often based on body surface area, with dose reductions in case of toxicity in subsequent cycles. It is, however, desirable to have the right dose from the first dose onward. Pemetrexed exposure correlates well with its toxicity (Latz et al., 2006) and, likely, its efficacy. It can be argued that microdosing can be used to predict the pharmacokinetics of pemetrexed during the first therapeutic dose, yet without pharmacological effect and,
thus, risks for the patient (van Nuland et al., 2019). Therefore, microdosing might be used to individualize the dosing of pemetrexed. Microdosing may also facilitate drug–drug interaction studies with this cytotoxic anticancer drug, studies that are frequently limited by the risk of toxicity. The present study reports the development of a highly sensitive bioanalytical assay to quantify pemetrexed in human plasma after a microdose (100 μg) for application in a pharmacokinetic study.

2 | MATERIALS AND METHODS

2.1 | Chemicals and materials

Pemetrexed (C_{20}H_{19}N_{5}Na_{2}O_{6}·7H_{2}O, cas. no. 357166-29-1) was purchased from Sigma-Aldrich (Amsterdam, The Netherlands), and its internal standard (IS) [^{13}C_{5}]−pemetrexed was purchased from Alsachim (Illkirch Graffenstaden, France). Formic acid (98−100%), acetonitrile U-LC/MS, methanol U-LC/MS, and n-hexane for analysis were purchased from Merck (Darmstadt, Germany). Ascorbic acid was purchased from Bufa (Jsselstein, the Netherlands). Water was obtained using a Purelab flex 4 system from Veolia (Ede, the Netherlands). Drug-free EDTA (ethylenediaminetetraacetic acid) anticoagulated whole blood was obtained from Sanquin (Nijmegen, the Netherlands) and was centrifuged for 5 min at 4300g at room temperature. The collected EDTA-plasma was filtered using a quantitative filter paper (474, medium filtration rate, particle retention 5–10 μm) and stored at −40°C.

2.2 | Preparation of stock solutions

Two separate pemetrexed stock solutions containing 4500 mg/L of pemetrexed in water and the IS stock solution containing 1000 mg/L of [^{13}C_{5}]-pemetrexed in water were stored at −40°C.

2.3 | Preparation of intermediate stock solutions

For the preparation of standards and internal quality control (QC) samples, the pemetrexed stock solutions were diluted with water to a concentration of 5.00 mg/L. The IS working solution had a concentration of 50 μg/L of [^{13}C_{5}]-pemetrexed in water. All solutions were stored at −40°C.

2.4 | Preparation of standards and internal QC samples

The concentration range for pemetrexed in plasma was set at 0.0250−25.0 μg/L, based on the expected pemetrexed concentrations associated with a 100-μg dose, as predicted using a Monte Carlo simulation of a previously developed population pharmacokinetic model (Latz et al., 2006). The first intermediate stock solution was diluted with filtered blank EDTA-plasma to achieve seven calibration concentration levels: 25.0 μg/L [HLOQ (higher limit of quantification)], 7.50, 2.50, 0.750, 0.250, and 0.0750 μg/L [alternative LLOQ (lower limit of quantification)], and 0.0250 μg/L (LLOQ).

The internal QC samples were prepared from the second intermediate stock solution resulting in concentration levels of 12.0, 1.20, 0.120, and 0.0600 μg/L in EDTA-plasma, designated as QC high, medium, alternative low, and low, respectively. The calibrators and internal quality samples were stored at −40°C.

2.5 | Sample preparation

Clinical samples consisting of EDTA-anticoagulated whole blood were centrifuged for 5 min at 1900g, and the resulting EDTA-plasma was stored at −40°C until further use.

2.6 | Solid-phase extraction method

New needle tips and solid-phase extraction (SPE) cartridges (30 μm, 60 mg, Oasis MAX, Waters [Etten-Leur, The Netherlands]) were placed on a vacuum manifold and rinsed with 0.5 mL of methanol and then with 0.5 mL of water. Next, 0.5 mL of water, 25 μL of IS working solution, and 1 mL of plasma (standard, QC sample or sample) were pipetted onto a cartridge when no pressure was applied. Subsequently, all samples were drawn into the cartridges by applying reduced pressure. The cartridges were then washed twice with 1 mL of water and twice with 1 mL of methanol. The analyze was eluted.
twice using 0.25 mL of methanol/formic acid (98:2, v/v) and reduced pressure and collected in a 10-mL glass tube with a short conical bottom. The eluent was evaporated to dryness under a nitrogen stream at 37°C. The residues were redissolved in 100 μL of acetonitrile:20 mg/mL of ascorbic acid:0.1% formic acid in water (2:1:7, v/v/v) and (washed with) 1 mL of n-hexane. The tubes were closed with a winged cap, mixed using a vortex mixer for 5 min, and centrifuged for 5 min at 4300g. Eighty microliters of the clear underlayer, without excess ascorbic acid on the conical bottom, was transferred into polypropylene autosampler vials with glass inserts. The vials were closed with a screw cap with pre-slit septum and centrifuged for 5 min at 1910g and 10°C.

2.7 | UPLC-MS/MS analysis
The Acquity H-class UPLC system consisted of a Quaternary Solvent Manager solvent delivery pump, a flow-through needle (FTN) autosampler, a column oven (CO), and a Xevo TQ-s micro tandem mass spectrometer. Ten microliters of the sample was injected into a BEH C18 column (100 × 2.1 mm i.d.; particle size, 1.7 μm) with a BEH C18 guard column (5 × 2.1 mm i.d.; particle size, 1.7 μm), and the CO temperature was set at 40°C. The mobile phase components were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The mobile phase started with 85% A and 15% B to elute pemetrexed for 3.5 min. From 3.6 to 4.5 min the column was rinsed with 35% A and 65% B. Then, the mobile phase returned to 85% A and 15% B to reequilibrate the column for 4.9 min. The flow rate was 0.3 mL/min, and the total run time was 9.5 min. The samples were stored at 10°C in the autosampler during analysis. The needle was washed post-injection for 6 s with a mixture of water and acetonitrile (90:10, v/v). The Xevo TQ-s micro mass spectrometer operated in the positive electrospray ionization mode using multiple reaction monitoring (MRM). The capillary voltage was 2.7 kV, source temperature was 150°C, desolvation temperature was 350°C, and the desolvation gas flow was set to 950 L/h. Argon was used as collision gas, and nitrogen was used as desolvation and nebulizer gas. The analyte and IS were optimized on the [M+H] ion, and one MRM transition was chosen for quantification and another for qualification. Proposed mass-to-charge ratio (m/z) fragmentation patterns for the analyte and its IS are shown in Figure 1. Cone voltage and collision energy were optimized for both components (Table 1). The source of the MS/MS detector of the UPLC-MS/MS, which was used for different assays during the week, was cleaned before every run. Also, with every injection only between 1.5 and 3.5 min the eluent was allowed into the source of the tandem mass spectrometer. Analytical runs were controlled and processed using Masslynx software version 4.1 (all by Waters, Etten-Leur, the Netherlands).

2.8 | Validation procedures
The validation of the assay in plasma was based on the most recent versions of the guidelines on bioanalytical method validation of the European Medicines Agency (EMA, 2011) and the U.S. Food and Drug Administration (FDA, 2018). In addition, we studied batch-to-batch difference in SPE cartridges, which is a validation parameter specifically related to SPE.

2.8.1 | Selectivity
Blank EDTA–plasma of six oncological patients who did not receive pemetrexed were evaluated for interference by endogenous substances.

The presence of interfering components was accepted if the response was less than 20% of the LLOQ for pemetrexed and less than 5% of the IS.

2.8.2 | Carryover
Carryover was assessed by injecting blank samples after the HLOQ. Carryover in the blank sample, after the HLOQ, had to be less than 20% of the LLOQ for pemetrexed and 5% of the IS.

2.8.3 | Accuracy and precision
Five replicates of samples of pemetrexed in EDTA-plasma at the LLOQ, alternative LLOQ, the four internal QC samples, and HLOQ were analyzed during three different days to determine accuracy and within-day and between-day precision of the method. To analyze these samples, seven calibration concentration levels were used in duplicate, in addition to the blank sample that was not incorporated in the calibration line. The calibration curve was constructed as a plot of the analyte concentration versus peak area ratio of the analyte to IS. The method of least squares was used to determine which regression model fitted the calibration data best. For each replicate measurement, the concentrations measured in the LLOQ, alternative

| Component         | Retention time (min) | Positive ion mode MRM transition trace (m/z) | Cone voltage (V) | Collision energy (eV) |
|-------------------|----------------------|---------------------------------------------|------------------|----------------------|
| Pemetrexed        | 2.71                 | 428.08 > 281.06                             | 14               | 18                   |
| Pemetrexed Q      | 2.71                 | 428.08 > 163.00                             | 14               | 36                   |
| [13C5]-Pemetrexed | 2.71                 | 433.10 > 281.01                             | 14               | 20                   |

Note: MRM, multiple reaction monitoring.
LLOQ, the four internal QC samples, and HLOQ samples were divided by the nominal concentrations. To assess accuracy, the mean ratio of measured concentrations versus nominal concentrations \( n = 15 \) was calculated and multiplied by 100.

One-way analysis of variance (ANOVA) was used to assess the within-day and between-day precision at each of the seven concentrations, using the run day as the classification variable. The error mean square or mean square within groups (ErrMS), the day mean square or mean square among groups (DayMS), and the grand mean (GM) of all 15 measurements across the three run days were obtained from the ANOVA. The estimate of the within-day and between-day precision at every concentration was calculated as follows:

\[
\text{Within – day precision} = \left( \frac{(\text{ErrMS})^{0.5}}{\text{GM}} \right) \times 100\%
\]

\[
\text{Between – day precision} = \left( \frac{[(\text{DayMS} - \text{ErrMS})/n]^{0.5}}{\text{GM}} \right) \times 100\%
\]

where \( n \) is the number of replicate measurements within each day.

For the LLOQ, the percentage deviation from the nominal concentration (accuracy) and the relative standard deviation (precision) had to be less than 20%, and for the alternative LLOQ, the four internal QC samples and HLOQ samples had to be less than 15%.

2.8.7 | Hemolyzed and lipemic plasma

The effect of hemolyzed plasma, with a H-Index (Lippi et al., 2018) of about 600, was investigated by analyzing five replicate samples at a QC high and extra low concentration.

The effect of lipemic plasma, with a concentration of 150 and 300 mg/dL purified soya bean oil (Intralipid 20%, Fresenius Kabi, Etten-Leur, the Netherlands), was investigated by analyzing five replicate samples at a QC low concentration. The percentage deviation between the mean concentrations as compared to the nominal value and the relative standard deviation in the measurement of each condition had to be less than 15%.

2.8.8 | Stability

The stability of the pemetrexed stock solutions at -40°C was tested when fresh stocks were prepared. The percentage deviation from the nominal concentration had to be less than 5%.

The stability during sample handling for the analyte in EDTA-plasma was verified by subjecting the samples to three freeze–thaw cycles, testing the stability at room temperature, 4–8 and -40°C. This was performed at four different concentration levels (QC high, medium, alternative low, and low) in spiked samples.

The stability of the processed samples in the autosampler was also tested. All processed samples obtained on the first day of assessment of accuracy and precision were reanalyzed after 2 days. For each sample, the percentage of concentrations obtained after 3 days in the autosampler compared to the initially measured concentration was calculated. For the LLOQ, the percentage deviation from the initially measured concentration (accuracy) and the relative standard deviation (precision) had to be less than 20%, and for the four internal QC samples and HLOQ samples, both these measures had to be less than 15%.

2.8.9 | Batch-to-batch difference in SPE cartridges

The batch-to-batch difference in the SPE cartridges was validated. Pemetrexed in QC high and low was analyzed fivefold with two different batches. The difference in mean concentrations measured with the two batches had to be less than 15%.

2.9 | Clinical sample analysis

We measured a pharmacokinetic curve at 0.5–1, 1–2, 3–4, and 6–8 h after the administration of a 100-μg dose, which was obtained as a
part of an ongoing clinical microdosing study with pemetrexed (ClinicalTrials.gov identifier: NCT03655834).

3 | RESULTS

3.1 | Selectivity

Plasma of six oncological patients without pemetrexed medication was evaluated and found to be free from potential endogenous or other interferences. Chromatograms of pemetrexed at the LLOQ level (0.0250 μg/L) and its blank, calibrator 3 (2.50 μg/L), and a study sample containing pemetrexed and IS are shown in Figure 2.

3.2 | Carryover

Carryover in the blank sample following the high-concentration calibrator proved not to be greater than 20% of the LLOQ and 5% of the IS.

3.3 | Calibration curve

The calibration curve for pemetrexed (peak area pemetrexed/peak area IS ratios versus concentrations) was constructed using a weighting factor of $1/x^2$ and was fitted quadratically. The regression coefficients ($r^2$) of all three calibration curves during validation of pemetrexed in EDTA-plasma were 0.9978 ± 0.0004.

3.4 | Accuracy and imprecision

The results of the analysis of five replicates of EDTA-plasma LLOQ, alternative LLOQ, the four internal QC samples, and HLOQ samples on three different days are presented in Table 2.

3.5 | Dilution integrity

The percentage deviation between the mean concentration of 1.5 times HLOQ after pipetting half or a quarter of the volume and the nominal concentration was 1.12 and 2.45%, respectively. The relative standard deviations after dilution were 2.27 and 1.48%.

3.6 | Matrix effect and recovery

The relative standard deviation of the MF calculated for QC low and QC high from the six lots is 2.18 and 2.31%, respectively. Recovery in duplicate at QC low concentration and at QC high concentration was 59 ± 1%. For the IS the recovery was 55 ± 5%.

3.7 | Hemolyzed and lipemic plasma

The percentage deviation between the mean concentration of QC low and QC high in hemolyzed plasma and the nominal concentration was 9.24 and 4.32%, respectively. The relative standard deviations in hemolyzed plasma were 5.25 and 2.00%.

The percentage deviation between the mean concentration of QC low in lipemic plasma with 150 and 300 mg/dL intralipid and the nominal concentration was 5.71 and 4.22%, respectively. The relative standard deviations in lipemic plasma were 5.04 and 3.26%.

3.8 | Stability

The results of the stability of pemetrexed in various solutions and at various circumstances in spiked samples are presented in Table 3. The processed samples of pemetrexed in plasma proved to be stable at 10°C after 2 days with mean concentrations of 102 ± 7.41% of the initial concentrations.
3.9 | **Batch-to-batch difference in SPE cartridges**

The difference in mean concentrations of pemetrexed in plasma for QC low and QC high measured with two batches of the SPE cartridges was 2.66 and 0.17%.

### Table 2

| Validation sample | Nominal concentration (µg/L) | Within-day accuracy (n = 5) (%) | Within-day imprecision (RSD%) | Between-day accuracy (n = 15) (%) | Between-day imprecision (RSD%) |
|-------------------|-------------------------------|-------------------------------|-------------------------------|----------------------------------|-------------------------------|
| LLOQ              | 0.0252                        | 74.2                          | 6.04                          | 77.4                             | 5.39                          |
| QC low            | 0.0602                        | 88.4                          | 4.03                          | 90.2                             | 0.780                         |
| Alternative LLOQ  | 0.0750                        | 107                           | 6.05                          | 103                              | 3.38                          |
| QC alternative low| 0.120                         | 90.6                          | 4.57                          | 98.6                             | 6.77                          |
| QC medium         | 1.20                          | 108                           | 8.79                          | 104                              | 3.99                          |
| QC high           | 12.0                          | 102                           | 2.86                          | 101                              | 0                             |
| HLOQ              | 25.2                          | 105                           | 5.93                          | 102                              | 3.23                          |
| Average           |                               | 96.5                          |                               | 96.5                             |                               |

Note: EDTA, ethylenediaminetetraacetic acid; HLOQ, higher limit of quantification; LLOQ, lower limit of quantification; QC, quality control; RSD, relative standard deviation.

### Table 3

| Matrix                  | Condition            | Time interval | Mean concentration compared to nominal concentration (%) |
|-------------------------|----------------------|---------------|--------------------------------------------------------|
| Stock solutions         | −40°C                | 24 months     | 102 ± 0.3 (n = 3)                                       |
| Spiked plasma           | Room temperature     | 7 days        | 99.3 ± 2.4 (n = 6)                                      |
|                         | 4°C                  | 7 days        | 94.9 ± 6.3 (n = 6)                                      |
|                         | −40°C                | 14 months     | 108 ± 4.8 (n = 6)                                      |
|                         | Three freeze–thaw cycles |          | 107 ± 5.5 (n = 6)                                      |
| Spiked whole blood      | Room temperature     | 3 days        | 97.9 ± 12.2a (n = 6)                                    |
|                         | 4°C                  | 3 days        | 102 ± 6.7 (n = 6)                                      |

aDisturbance in chromatogram due to hemolysis.

3.10 | **Clinical sample analysis**

A plasma concentration–time curve of pemetrexed in one patient after the administration of a single dose of 100 µg of pemetrexed is shown in Figure 3. This dose is approximately 10,000-fold lower than a therapeutic dose.

4 | **DISCUSSION**

4.1 | **Development**

The development of an ultrasensitive assay, fit for microdosing, was accompanied with several specific analytical challenges.

4.1.1 | **Mass-to-charge ratio (m/z) fragmentation patterns**

Acquisition parameters were optimized using Intellistart (Waters). The two best mass-to-charge ratios (m/z) for pemetrexed were used during validation The m/z ratio of 428.08/281.06 yielded better sensitivity and less disturbance than the m/z ratio of 428.08/163.00 (Figure 1), which resulted in the first m/z ratio to be chosen for quantification and the second m/z ratio for qualification. The best m/z for [13C5]-pemetrexed, the IS, was 433.10/281.01.

4.1.2 | **Chromatography**

The pH of the eluent had an influence on the sensitivity of the assay. A lower pH of 3 yielded more response and less noise than a pH of 7. The BEH C18 column yielded a sufficient response for pemetrexed, just as the silica-based HSS T3 column and the BEH phenyl column (Waters). As the HSS T3 column and the BEH phenyl column are less rugged and the BEH phenyl column had also a broad and tailing peak shape, the BEH C18 column was chosen.
The retention time of pemetrexed was 2.8 min, but because there was no clear baseline after pemetrexed eluted, a rinsing step followed by reequilibration was needed before injecting a new sample.

Further, the addition of more water to the FTN wash solvent with water and acetonitrile (90:10, v/v) did not reduce the carryover effect after injection sufficiently. By decreasing the amount of the IS to a final concentration of 12.5 μg/L in every sample, the carryover met the criteria of less than 20% of the LLOQ and 5% of the IS.

4.1.3 | Sample pretreatment

The sample pretreatment was exploited to improve assay sensitivity. Because pemetrexed is a very polar compound and tends to remain in the water phase, SPE was chosen instead of liquid–liquid extraction. Further, 1 mL of sample was needed to quantify the extra LLOQ. By redissolving in 100 μL of solution after SPE, the sensitivity was increased 10 times. As the pKₐ values for the two carboxylic acid moieties of pemetrexed are 3.6 and 4.4 (PubChem Pemetrexed compound summary, 2019), a clean baseline and a stable recovery for pemetrexed were achieved with the Oasis MAX sorbent for acids, with pKₐ 2–8. The Oasis 2 × 4 method guide suggests a first wash step with 5% ammonia, but higher recovery was achieved by washing with water. After the sample was redissolved, the solution was not quite clear and caused the column to crash. When the sample was centrifuged for 5 min, a precipitate was visible at the bottom of the tube, but still the solution was not clear to inject. By adding a rinsing step with n-hexane during redissolving, a clear underlayer could be transferred to a polypropylene autosampler vial with glass insert.

4.1.4 | Type of plasma

Initially, citrate plasma was used for standards and internal QC samples during method development.

Then, validation samples were prepared in defrosted Li–heparin plasma to match possible future patient samples. However, freezing of Li–heparin plasma resulted in the formation of lumps that obstructed the SPE cartridge material. Therefore, the flow in the SPE cartridges decreased dramatically.

Processing defrosted EDTA–plasma of oncology patients who did not receive pemetrexed maintained the flow in the SPE cartridges constant. Therefore, validation samples in defrosted pooled drug-free EDTA–plasma, obtained from several blood tubes with the same blood type from the blood bank, were prepared. Again, the flow in the SPE cartridges decreased dramatically when the validation samples in EDTA–plasma were processed.

Also, less response of pemetrexed and its IS was obtained in processed (Li–heparin and) EDTA–plasma than in citrate plasma. This meant that standards and internal QC samples had to be prepared in the same type of plasma as our future samples.

Finally, when the pooled EDTA–plasma was filtered using a quantitative filter paper before making standards and internal QC samples, the problem of decreased flow in the SPE cartridges after pipetting plasma was solved.

4.1.5 | Carryover during sample pretreatment

Because of the low concentration range and high dynamic range needed in this assay, a substantial carryover effect was observed for pemetrexed during SPE.

In our conventional vacuum manifold up to 24 cartridges could be used simultaneously to extract our samples. When a second batch of 24 new cartridges was extracted, concentrations in the blank were found up to 50% higher than LLOQ after extracting HLOQ at the same position in the first batch.

A higher elution volume or adding water to the elution solution did not reduce carryover.

Rinsing the Luer fittings on the manifold lid after every extraction batch of 24 cartridges with water and ethanol and then drying with pressed air before using it for the next batch also did not help.

When new long needle tips (Waters) were used for every standard, QC or sample, instead of the reusable Luer fittings, the carryover problem was solved.

4.1.6 | Stability in processed samples

The last hurdle was attributed to the stability of pemetrexed in processed samples. Earlier, it was observed that pemetrexed undergoes degradation through oxidation and hydrolysis (Jansen et al., 2016). Cooling the autosampler to 10°C did not provide sufficient time to keep pemetrexed stable during quantification. Earlier
research showed that adding ascorbic acid prevents oxidation and therefore improves the stability of rifampicin after processing (Kolmer et al., 2017). The addition of ascorbic acid (BUFA, IJsselstijn, the Netherlands) resulted in improved processed sample stability for 2 days at 10°C.

4.2 Validation procedures: accuracy and precision

Because of the low concentration range needed in this assay two LLOQs and two QC lows were validated, where the concentration of QC low had to be less than thrice LLOQ, according to EMA guidelines on bioanalytical method validation. The LLOQ of 0.025 μg/L was based on the lower limit of the expected pemetrexed concentration associated with a 100-μg dose (2.4), and its QC low was 0.06 μg/L. The concentration of 0.075 μg/L (calibrator 6) was named alternative LLOQ, and its QC low (0.12 μg/L) was named alternative QC low.

In the range of 0.0600–25.0 μg/L we show that the method is accurate and precise, with a maximum coefficient of variation of 8.80%, and complies with the guidelines on bioanalytical method validation of the EMA (EMA, 2011) and the FDA (FDA, 2018).

Although the accuracy of 77% at the LLOQ level of 0.0250 μg/L did not comply with the preset requirements (80–120%), we decided to accept this potential disadvantage of our assay, as the lower concentrations were expected to be observed at the end of a concentration–time curve and have only a limited contribution for the calculation of the cumulative exposure.

4.3 Clinical sample analysis

All measured concentrations of the pharmacokinetic curve after a microdose were within the validated range, although some samples had to be diluted before quantification.

5 CONCLUSION

We successfully developed a highly sensitive UPLC-MS/MS assay for the analysis of pemetrexed in EDTA-plasma after microdosing. To the best of our knowledge, this is the first bioanalytical assay to quantify these low concentrations of pemetrexed.

We here show the feasibility of using a widely available UPLC-MS/MS equipment to measure low concentrations of pemetrexed to support a clinical microdosing study using this drug. These results will be published in a separate manuscript.

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CONFLICT OF INTEREST

All authors declare that they have no actual or potential conflicts of interest, including any financial, personal, or other relationships with other people or organizations, that could inappropriately influence, or be perceived to have influenced, this work.

AUTHOR CONTRIBUTIONS

E.W.J.E.-B.K. was involved in methodology, validation, formal analysis, writing—original draft, and investigation.

M.J.A.T. was involved in methodology and writing—review and editing.

R.J.B. assisted with writing—review and editing and resources.

N.R. helped with writing—review and editing and resources.

J.A.B. helped with writing—review and editing and resources.

R.H. was involved in conceptualization, funding acquisition, writing—review and editing, and project administration.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Rob ter Heine https://orcid.org/0000-0003-2185-8201

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