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Short communication

Development of an ion-pairing reversed-phase liquid chromatography method using a double detection analysis (UV and evaporative light scattering detection) to monitor the stability of Alimta®-pemetrexed preparations: Identification and quantification of L-glutamic acid as a potential degradation product

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ABSTRACT

A new method based on high-performance liquid chromatography coupled to ultraviolet and evaporative light scattering detection (HPLC-UV-ELSD) was developed for the determination of L-glutamic acid, a potential degradation product of pemetrexed, and for the quantification of pemetrexed itself. This is an ion-pairing, reversed-phase method. The column was a Synergi MAX-RP C12 4 μm (150 mm × 4.6 mm). The mobile phase was 1 mM tridecafluoroheptanoic acid in aqueous solution and acetonitrile under gradient elution mode. L-Glutamic acid was detected by ELSD, and pemetrexed by UV at 254 nm. Good resolution was achieved between pemetrexed and L-glutamic acid. The HPLC method was validated according to SFSTP and ICH guidelines, and applied the accuracy profile procedure with a five-level validation experimental design. For pemetrexed, the decision criteria selected consisted of the acceptability limits (±3%) and the proportion of results within the calculated tolerance intervals (95%). In conclusion, the proposed analytical procedures were validated over the selected validation domains for L-glutamic acid (0.005–0.025 mg/mL) and pemetrexed (0.4–0.6 mg/mL) and shown to provide a very effective method.

1. Introduction

Pemetrexed disodium has the chemical name L-glutamic acid, N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1*H*-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl] disodium salt, heptahydrate (Fig. 1A) and is a multitargeted antifolate that has demonstrated antitumor activity against various tumor types, both as a single agent and in combination with other chemotherapeutic agents [1,2]. Pemetrexed is used in the treatment of malignant pleural mesothelioma in combination with cisplatin in patients with unresectable disease, or for whom curative surgery is not an option [3,4]. It is also approved as a second-line, single-agent treatment for locally advanced or metastatic non-small cell lung cancer (NSCLC) [2]. The role of pemetrexed as maintenance therapy after first-line therapy for advanced NSCLC has been evaluated in a phase-III trial [5]. Pemetrexed is currently registered for first-line therapy in combination with cisplatin, at a dose of 500 mg/m^2, diluted in 0.9% physiological saline, on day 1 of a 3-week schedule, and, as a single-agent for the second-line treatment of patients with non-squamous NSCLC [6].

Although the stability of pemetrexed solutions for intravenous administration has been extensively studied [7–9], there are no extensive data available concerning the identification and/or quantification of the potential degradation products. Moreover, analytical methods described in the literature, such HPLC coupled with UV detection (recommended by the European Pharmacopoeia [10]), may not be specific enough for this type of analysis when the drugs or the degradation products, such as most amino acids, are lacking a chromophore [11,12].

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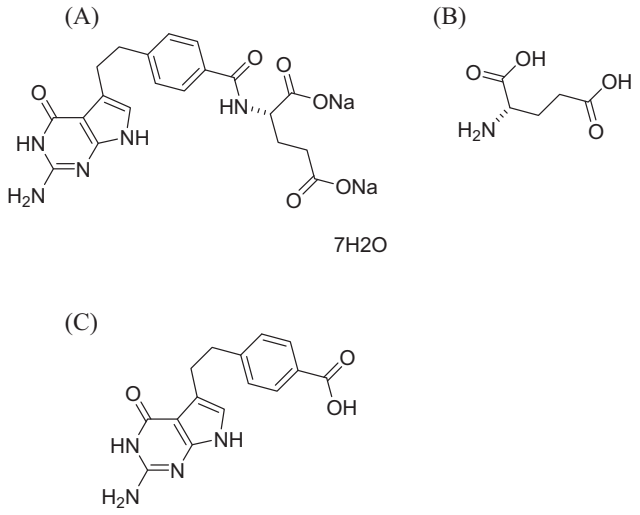

Fig. 1. Chemical structures of pemetrexed (A), L-glutamic acid (B), and impurity A (C).

Saravanan et al. have developed a method with UV detection based on the quantification of pemetrexed (Fig. 1A) and the detection of impurity A (Fig. 1C), a potential degradation product [11]. Nevertheless, this method is not exhaustive since L-glutamic acid is also a potential degradation product of pemetrexed, and indeed pemetrexed is obtained by peptide chemistry between the carboxylic moiety of impurity A and the amine group of L-glutamic acid [13]. We have therefore focused our analysis on L-glutamic acid (Fig. 1B) *versus* impurity A, because they should appear in a 1/1 ratio, and so this could provide some indication of the degradation pathway of pemetrexed.

L-Glutamic acid is a polar molecule with no strong chromophore group (weak absorbance near 220 nm), and several studies have been published on the use of ELSD or Corona detector to determine polar underivatized amino acids [14–16]. Furthermore ELSD is viewed as suitable for the detection for non-absorbing analytes [17]. The chromatographic mobile phase is nebulized with an inert gas, and evaporated in a drift tube. The response does not depend on the optical properties of the solute, and any compound less volatile than the mobile phase can be detected. This method can be used advantageously to search for the degradation products of pharmaceutical products. Moreover to obtain structural information about the degradation products formed, the chromatographic conditions developed using ELSD detection could be transposed directly to an HPLC-MS^n method, as these two detection modes share the same chromatographic requirements (essentially a volatile mobile phase).

The objective of this study was to develop an HPLC method for determining stability that is able to separate pemetrexed, Na^+, L-glutamic acid and impurity A, which could be used to quantify pemetrexed and L-glutamic acid, a commercially available by-product, and to separate and detect all potential degradation products. Furthermore, the method described here could be used for routine stability studies of pemetrexed in hospital pharmacies.

2. Experimental procedure

2.1. Chemicals and reagents

Alimta®-pemetrexed (Fig. 1A) was purchased from the hospital pharmacy at the Tours Teaching Hospital (Lilly, local preparation). HPLC-grade acetonitrile and methanol were purchased from Carlo-Erba (Val de Reuil, France). Trifluoroacetic acid (TFA, 99% purity), nonafluoropentanoic acid (NFPA, 97% purity), and tridecafluoroheptanoic acid (TDFHA, 99% purity) were purchased from Sigma Aldrich (St Quentin Fallavier, France) as was L-glutamic acid (Fig. 1B). Ultra-pure water produced by a Millipore (Molsheim, France) system was used throughout. Impurity A was synthesized in our laboratory in six steps, and used as a 0.5 mg/mL solution in methanol [13].

2.2. Instrumentation

HPLC analyses were carried out with a LaChrom Elite system equipped with a VWR L-2130 pump, a VWR L-2200 autosampler at 4 °C, a VWR L-2400 UV detector from Merck (Fontenay sous bois, France) and a SEDEX 85 evaporative light scattering detector (ELSD) purchased from SEDERE (Alfortville, France). The chromatographic data handling was accomplished using EZChrom Server software (Merck, Darmstadt, Germany). The UV detection was carried out at 254 nm. The usual ELSD settings were as follows: photomultiplier, 10; evaporative tube temperature, 50 °C; air pressure, 3.5 bar.

The column was a Synergi MAX-RP C12 column (Phenomenex, 4 μm, 150 mm × 4.6 mm) from Phenomenex (Le Pecq, France) connected to a guard-column MAX-RP (4 mm × 3 mm), thermostated at 20 °C.

The mobile phase was filtered and degassed before use. A gradient of eluent A (TDFHA 1 mM in aqueous solution) and eluent B (acetonitrile) was established as follows: 85% (v/v) eluent A over 6 min, then to 70% (v/v) eluent A over 28 min and, finally, a return to the initial conditions in 0.1 min. The flow rate throughout was 1 mL/min.

2.3. Procedures

2.3.1. Quantification

2.3.1.1. Pemetrexed. Alimta®-pemetrexed was dissolved in 0.9% physiological saline (NaCl 0.9%) solution to obtain a concentration of 25 mg/mL. This solution was then diluted with the mobile phase to provide an intermediate solution containing 1 mg/mL of pemetrexed (solution A). UV detection at 254 nm was used to quantify pemetrexed.

2.3.1.2. L-Glutamic acid. This amino acid was dissolved in water to obtain a 0.1 mg/mL stock solution (solution B). ELSD detection was used to quantify L-glutamic acid.

An external standard calibration curve with five calibration points of L-glutamic acid and pemetrexed was obtained with mixtures of solution A and solution B diluted in the mobile phase. For L-glutamic acid, the calibration points ranged from 0.005 to 0.025 mg/mL (0.005, 0.010, 0.015, 0.020, 0.025 mg/mL) and for pemetrexed, they ranged from 80% to 120% of the assay concentration (0.4, 0.45, 0.5, 0.55, 0.6 mg/mL). The point at 0.005 mg/mL of L-glutamic acid was near to the limit of detection that we wanted to achieve (1% (w/w) for monitoring the degradation of a solution of pemetrexed).

2.4. Method validation

The method was validated according to SFSTP and ICH guidelines [18–22].

2.4.1. Selectivity

The selectivity of the method was established by determining the resolution of the pemetrexed peak from those of the potential degradation products, L-glutamic acid and impurity A.

Table 1
Influence of the perfluorinated acid nature and the acetonitrile percentage in mobile phase on the resolution between L-glutamic acid and sodium ion.

Mobile phase (v/v)	Retention time of L-glutamic acid (min)	Retention time of the sodium ion (min)	Resolution
NFPA 6.2 mM/ACN (100/0)	10.3	6.9	2.3
NFPA 6.2 mM/ACN (99/1)	10.2	6.9	2.4
NFPA 6.2 mM/ACN (98/2)	8.8	7.1	1.3
NFPA 6.2 mM/ACN (85/15)	4.2	3.7	0.8
TDFHA 1 mM/ACN (90/10)	8.5	23	7.4
TDFHA 1 mM/ACN (85/15)	**4.0**	**13.6**	**9.6**
TDFHA 1 mM/ACN (80/20)	3.0	6.8	5.1
TDFHA 1 mM/ACN (70/30)	1.9	2.8	1.5

Optimized mobile phase conditions are shown in bold.

2.4.2. Precision

Three injections of five different solutions (containing 0.4, 0.45, 0.5, 0.55, 0.6 mg/mL of pemetrexed and 0.005, 0.010, 0.015, 0.020, 0.025 mg/mL of L-glutamic acid), were carried out on the same day, and the values of the relative standard deviation (RSD) were calculated to determine the intra-day precision. These determinations were also repeated on three different days to determine the inter-day precision.

The inter-day precision of the retention time of the L-glutamic acid was also evaluated.

2.4.3. Accuracy profile

The following experimental design was applied: five levels and two replicates over a 3-day period for the calibration standards, and five levels and three replicates over a 3-day period for validation standards. All calibration standards and validation standards were independent.

2.4.4. Limit of detection

This value for L-glutamic acid was estimated mathematically from the standard curve equation. The LOD was calculated based on the concentration exhibiting a signal-to-noise ratio of 3 [22].

2.4.5. Stability

The stability of pemetrexed and L-glutamic acid was studied under different conditions:

- One set of samples ($n = 3$) of the stock solutions was stored at +2 to +8 °C for 48 h. The stability of the stock solutions was assessed by duplicate determinations of samples diluted immediately on day 0 (containing 0.5 mg/mL pemetrexed and 0.015 mg/mL L-glutamic acid), and 24 and 48 h later.
- The post-dilution stability (i.e. stability in the autosampler) was assessed by duplicate determinations of samples (containing 0.5 mg/mL pemetrexed and 0.015 mg/mL L-glutamic acid; $n = 3$) immediately after dilution, and after 24 h and 48 h in the injector.

Finally, different concentrations of 0.9% physiological saline (NaCl 0.9%) were added to the sample analyzed to evaluate their influence on the quantification of pemetrexed (0.5 mg/mL) and of L-glutamic acid (0.005 mg/mL) in the concentration assay.

3. Results and discussion

3.1. Development of the chromatography method

The goal of our work was to identify a chromatographic system that would be able to exclude L-glutamic acid, a highly polar compound, from the void volume, to separate this compound from sodium ions, and to elute both pemetrexed and impurity A within an acceptable analysis time (less than 35 min). The pemetrexed solution was reconstituted and diluted in the hospital pharmacy using 0.9% physiological saline (NaCl 0.9%), which meant that sodium and chloride were both present in all the samples analyzed. Sodium is a non-volatile cation that can be detected by ELSD, whereas chloride is a semi-volatile anion, and consequently poorly detected by ELSD. Moreover, the fact that ELSD was necessary to detect L-glutamic acid, a solute with no strong chromophore group, made it impossible to use any non-volatile reagents in the mobile phase. The methods previously described for the quantification of pemetrexed in liquid pharmaceutical preparations were not compatible with these requirements, as they use either non-volatile salts (phosphate buffer) in the mobile phase [7,11] or acetic acid, which is a volatile reagent, but one unsuitable for the retention of L-glutamic acid [7–9].

In an attempt to obtain good separation between pemetrexed, L-glutamic acid, sodium ions and impurity A, we used a Synergi MAX-RP C12, 4 μm column. This column has lower steric hindrance than a C18 column, and covers 25% more of the silica surface, thus shielding more free silanols. Moreover, due to its high specific surface area (475 m^2/g), the Synergi MAX-RP gives similar hydrophobic retention and methylene selectivity to a C18 column, but with sharper peaks, less peak tailing, and excellent reproducibility [23]. For the mobile phase, various perfluorinated carboxylic acids (TFA, NFPA, TDFHA) were tested as ion-pairing reagents in the mobile phase [14,15]. We first evaluated the acidic mobile phase using 0.1% trifluoroacetic acid–acetonitrile (85:15), and found that under these conditions L-glutamic acid was not retained on the column. When TFA was replaced by NFPA in the mobile phase, this did not provide sufficient resolution between sodium ions and L-glutamic acid (Table 1). Finally, we resolved this problem by using a mobile phase consisting of a combination of 1 mM tridecafluoroheptanoic acid aqueous solution and acetonitrile (85:15), which gave the best resolution between sodium ions and L-glutamic acid, with a good and reproducible retention time (4 min) (Tables 1 and 2). As shown in Table 1, the length of the carboxylic chain of the perfluorinated carboxylic acid influences the retention of sodium ions more than that of L-glutamic acid. In order to elute pemetrexed and impurity A within an acceptable analysis time, it was then necessary to carry out 15–30% acetonitrile gradient elution over 28 min, starting 6 min after the beginning of the run. For routine quantification experiments, the injection sequence started with two blank injections, which were subsequently discarded, in order to equilibrate the column. An equilibration time of 21 min was allowed between successive injections.

Fig. 2 shows the optimized LC-UV-ELSD analysis of a solution of pemetrexed (0.5 mg/mL) spiked with 0.005 mg/mL L-glutamic acid and 0.1 mg/mL impurity A. Pemetrexed and impurity A were detected by both detectors, but L-glutamic acid and Na^+ could only be detected by ELSD. Under the conditions described above, the retention time for L-glutamic acid was about 4 min; pemetrexed and impurity A were eluted at about 30 and 34 min, respectively (Fig. 2). The modification of the baseline observed between 24 and 28 min is due to acetonitrile gradient elution and associated

Table 2
Validation results for the determination of pemetrexed (mg/mL) (A) and L-glutamic acid as a potential degradation product (mg/mL) (B).

Validation criterion	Level 1	Level 2	Level 3	Level 4	Level 5
A					
Mean introduced concentration	0.40	0.45	0.50	0.55	0.60
Mean predicted concentration	0.397	0.443	0.494	0.549	0.597
Absolute bias	−0.003	−0.007	−0.006	−0.001	−0.003
Relative bias (%)	−0.75	−1.57	−1.14	−0.24	−0.43
Recovery (%)	99.3	98.4	98.9	99.8	99.6
Repeatability standard deviation	0.002	0.001	0.001	0.001	0.001
Intermediate precision standard deviation	0.003	0.001	0.002	0.003	0.003
Repeatability RSD (%)	0.50	0.15	0.21	0.17	0.20
Intermediate precision RSD (%)	0.86	0.17	0.33	0.51	0.50
Lower β tolerance limit	0.385	0.441	0.489	0.536	0.585
Upper β tolerance limit	0.409	0.445	0.500	0.561	0.610
Lower relative β tolerance limit (%)	96.3	98.0	97.8	97.5	97.4
Upper relative β tolerance limit (%)	102.2	98.9	99.9	102.1	101.7
B					
Mean introduced concentration	0.005	0.010	0.015	0.020	0.025
Mean predicted concentration	0.0050	0.0097	0.0148	0.0200	0.0260
Absolute bias	0.00004	−0.00031	−0.00016	0.00003	0.00104
Relative bias (%)	0.72	−3.09	−1.10	0.14	4.14
Recovery (%)	100.7	96.9	98.9	100.1	104.1
Repeatability standard deviation	0.00017	0.00027	0.00023	0.00032	0.00024
Intermediate precision standard deviation	0.00019	0.00032	0.00023	0.00032	0.00068
Repeatability RSD (%)	3.3	2.8	1.6	1.6	0.9
Intermediate precision RSD (%)	3.9	3.3	1.6	1.6	2.5
Lower β tolerance limit	0.0045	0.0088	0.0143	0.0192	0.0230
Upper β tolerance limit	0.0056	0.0106	0.0154	0.0208	0.0290
Lower relative β tolerance limit (%)	90.3	88.4	95.1	96.2	92.1
Upper relative β tolerance limit (%)	111.1	105.5	102.7	104.1	116.2
Mean retention time (min)	4.014	4.011	4.004	3.998	3.994
Intermediate precision standard deviation (min)	0.011	0.011	0.010	0.010	0.013
Intermediate precision RSD (%)	0.28	0.28	0.26	0.27	0.33

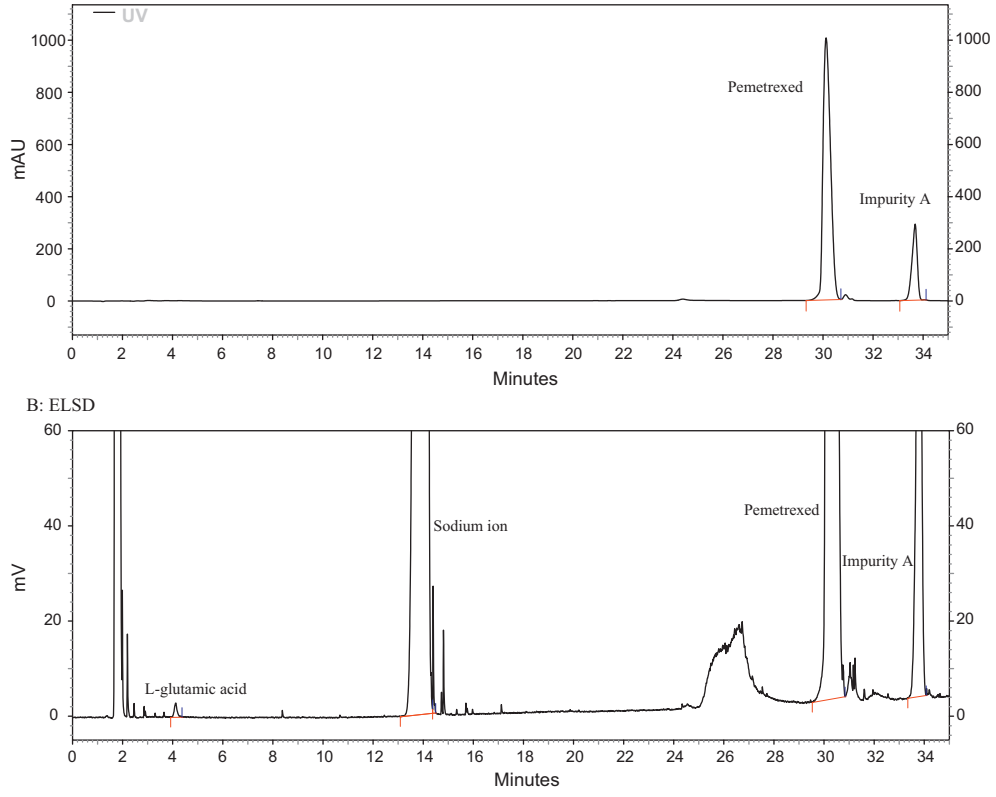

Fig. 2. HPLC analysis of a solution of pemetrexed (0.5 mg/mL) spiked with L-glutamic acid (0.005 mg/mL) and impurity A (0.1 mg/mL). Column: Synergi MAX-RP C12 4 μm (150 mm × 4.6 mm I.D.). Gradient elution: solvent A: 1 mM TDFHA in water, solvent B: acetonitrile; 0–6 min 15% B, 6–34 min linear gradient to 30% B. Flow rate: 1 mL/min; injection volume: 20 μL; 2 A: UV detection at 254 nm. 2 B: ELSD detection (P = 3.5 bar; T = 50 °C; Gain 10).

to a system peak. It has been previously demonstrated that the ion-pairing agent added to mobile phase induces a dynamic modification of the surface of the reversed-phase packing material [15]. So, by increasing the acetonitrile percentage in the mobile phase, some amounts of TDFHA, hydrophobic agent which is more soluble in ACN than in water, were desorbed from the stationary phase and migrated towards the detector. This phenomenon can be observed with ELSD due to differences in mobile phase volatility when TDFHA concentration increases suddenly. The proposed method therefore provides satisfactory separation of the drug (pemetrexed) from its potential degradation products (L-glutamic acid and impurity A). It is important to point out that all the methods published hitherto for this drug [7–9,11] have involved the use of UV detection of the degradation products at 254 nm, and so are non-exhaustive methods, because some of its degradation products do not absorb in UV (e.g. L-glutamic acid), and so would not be detected. ELSD is able to detect such products, and moreover, it is already being used in several methods for detecting impurities in pharmaceutical products [17,24–25], which is an advantage for the development of an HPLC method suitable for determining drug stability.

3.2. Method validation

3.2.1. Precision

The data obtained from the intra- and inter-day and retention time precision experiments for pemetrexed and L-glutamic acid are shown in Table 2. The RSD values for the intra-day and inter-day precision of the quantification of pemetrexed were <1%. For L-glutamic acid, the RSD values for the intra-day and inter-day precision were <4.0%, and the inter-day precision of its retention time was <1%. The method is therefore sufficiently precise for stability studies (Table 2).

3.2.2. Linearity of the response

Regarding the calibration data, the UV response for pemetrexed was strictly linear in the concentration range from 0.4 to 0.6 mg/mL; the ELSD response for L-glutamic acid has been shown to be linear on double logarithmic coordinates in the concentration range from 0.005 to 0.025 mg/mL. Knowing the theoretical concentrations of the samples, the trueness and tolerance intervals were computed for each concentration of pemetrexed and L-glutamic acid (Table 2). The accuracy profiles are shown in Fig. 3.

For pemetrexed, the tolerance interval was within the ±3% acceptance limit at all concentrations except at 0.4 mg/mL (80% of the assay concentration), where the lower acceptance limit value found was 96.3%. This validation provides sufficient guarantee that the method will provide results ±3% of the true value in at least 95% of cases, when the concentration is between 0.45 and 0.6 mg/mL (i.e. from 90% to 120% of the concentration assay). Despite the lower acceptance limit at 80% of the assay concentration, this method is therefore validated for stability studies. Indeed, it is accepted that a drug can be defined as being stable if not less than 90% of the initial drug concentration remains in the solution [7].

For L-glutamic acid, the tolerance interval was within the ±15% acceptance limit at all concentrations. This validation provides sufficient guarantee that the method will provide results at ±15% of the true value in at least 95% of cases, when the concentration is between 0.005 and 0.025 mg/mL (i.e. from 5% to 20% (w/w) degradation of a 0.5-mg/mL solution of pemetrexed (assay concentration)). The limit of detection was calculated to be 0.0018 mg/mL, so the method should detect 1.5% (w/w) degradation of a 0.5 mg/mL solution of pemetrexed (assay concentration).

3.2.3. Stability and influence of the dilution solvent

Pemetrexed and L-glutamic acid both remained stable under the conditions studied (CV < 2%), and the concentration of NaCl 0.9% did not influence the quantification of L-glutamic acid or pemetrexed at the concentrations studied (data not shown).

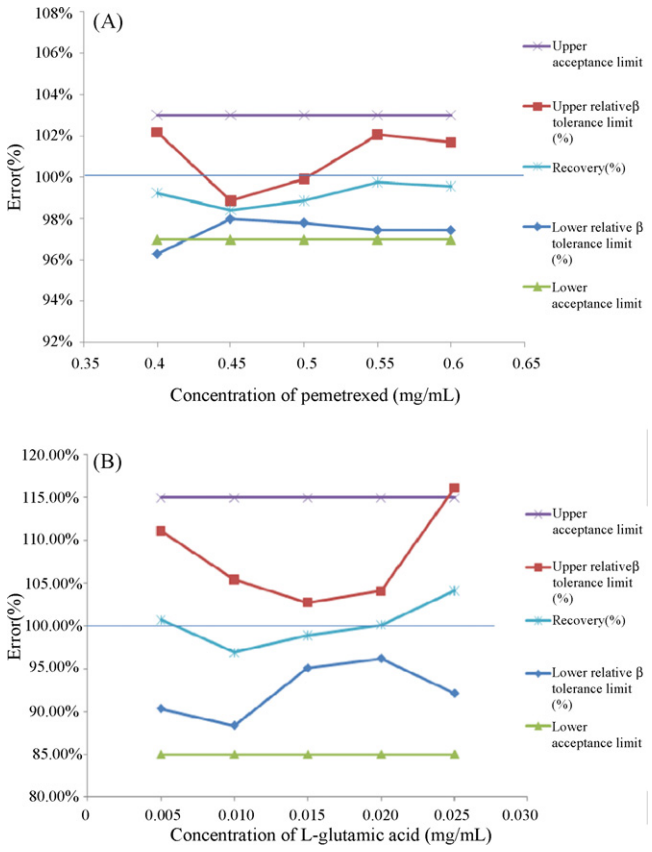

Fig. 3. Pemetrexed determination in mg/mL (acceptance limits ±3%) (A) and L-glutamic acid determination in mg/mL (acceptance limits ±15%) (B).

4. Conclusion

As we have stated, it is accepted that a drug can be classified as being stable if not less than 90% of the initial drug concentration remains in solution if the degradation products are less toxic than the drug itself, or not less than 95% of the initial drug concentration remains in solution if the degradation products are more toxic than the drug. The method described here can detect a 1.5% level of a potential non-toxic degradation product, and so could also be used to provide some indication of the degradation pathway of pemetrexed. L-Glutamic acid is known to be non-toxic – but what about impurity A and all the other potential degradation products? The HPLC-UV-ELSD method could be combined with MS^n detection to confirm the degradation pathway of pemetrexed under conditions of hospital use, and to give an indication of the structure of the degradation products other than L-glutamic acid and impurity A. Moreover, this ion-pairing HPLC-UV-ELSD method could be used for assessing the stability of solutions of pemetrexed in hospital centers.

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